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CHOLERA

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Preface

It would be no exaggeration to say that it was through cholera, and the fear to which its pandemic sweeps gave rise, that international solidarity in matters of health was born Cholera was the principal disease covered by the early international sanitary conventions and came at the head of the list of quarantinable diseases Koch s discovery of the cholera vibrio and thus the confirmation of the contagion theory in 1884 was a scientific keystone of the greatest importance and lay at the base of much progress in the drafting of future sanitary conventions While in Europe cholera has not been seen since the early twentieth century its endemic foci in Asia remain occasionally erupting into epidemics The disease continues to claim an annual toll of tens of thousands on that continent and to menace other parts of the world as witnessed by the 1947 epidemic in Egypt whence the disease had disappeared since 1919

Much has been written on cholera in the past hundred years, but a great part of the work is scattered among the periodicals of the world and is often not easily accessible Some of this work has proved to be of tran sitory importance some on the other hand now fallen into neglect, merits inclusion in the history of medical discovery To-day research workers are still at grips with a number of problems among them the immunological characteristics of the vibrio and their implications for cholera vaccine differential bacteriological and biochemical diagnosis and phage typing and a variety of practical questions of prevention and treatment depending on these

To assist public health services responsible for cholera control in endemic areas and to provide guidance for those who may one day be faced with the problem in countries now free from the disease the World Health Organization invited Dr R. Pollitzer to prepare a monograph on cholera The eleven chapters which constitute the main part of this book originally appeared as separate articles in the *Bulletin of the World Health Organization*

ACKNOWLEDGEMENTS

During the three years which he devoted to the compilation of the present book, the author has had the benefit of much assistance advice and encouragement from many institutions and persons. Impossible as it is to enumerate all of them, he wishes nevertheless to record his particularly great indebtedness to the following The Regents of the University of California most generously accorded to the writer the status of a research associate attached to the George Williams Hooper Foundation Medical Center San Francisco The director of this institution, Dr K F Meyer and also Dr B Eddie not merely granted him adequate facilities for writing the text but were indefatigable in helping the author in every possible way and constantly encouraging him Most kindly given help in administrative matters was received from Professor H G Johnstone, Dean of Students in the Medical Center Even so it would have been impossible to complete the work had not the author been given most generous grants in-aid first from the Division of Research Grants and Fellowships of the National Institutes of Health, US Department of Health Education, and Welfare then by the Foundation for Microbiology Rutgers University New Brunswick, N.J., USA, and finally by the World Health Organization.

The writer s great responsibility in compiling this monograph has been much alleviated by the willingness of two colleagues outstanding in cholera research to participate in his labours Dr Satya Swaroop who combines a most authoritative knowledge of medical statistics with a thorough acquaintance with the cholera problem in India, kindly consented to be mainly responsible for the part of the work dealing with the present incidence of the disease similarly Dr William Burrows put the author under a deep obligation by participating in the most difficult task of discussing the problems of cholera immunology to the elucidation of which his own researches have so much contributed. The author is also much indebted to Dr Jean Gallut of the Institut Pasteur Paris, who has undertaken for the World Health Organization the translation into French of this book, for constantly furnishing information on his own important cholera research work.

It might seem at first glance that San Francisco now far in time as well as in space from any cholera manifestation would hardly be a proper locale for making a study of the problems of this disease Actually however the rich main library of the Medical Center in combination with the literary treasures possessed by the Hooper Foundation furnished the author with most of the information which he needed while certain series of publications, which could not be found on the campus, were fortunately within easy reach in the Lane Library of Stanford University San Francisco The author has to thank the staffs of these libraries not only for permission to consult their files, but also for going far out of their way to make these studies particularly easy and enjoyable On the comparatively rare occasions on which help was required from libraries outside San Francisco the needs of the author were most obligingly responded to by the Library of the World Health Organization in Geneva or in a few instances, by the National Library of Medicine (formerly Surgeon General s Library) in Washington D C

over a period of three years they have now been revised and brought up to date in the light of comments received and new information made available during that period.

Dr Pollitzer writes with an authority derived from a long career devoted largely to the fight against cholera and plague His monograph on the latter disease published by WHO in 1954 is already recognized as a classic the World Health Organization publishes the present work in the confident hope that it will receive the same acclaim.

Chapter 1

HISTORY OF THE DISEASE

Earliest Evidence

The evidence adduced to prove that epidemic or, as it is commonly called, Asiatic cholera, a specific infection caused by the *Vibrio cholerae* was present in ancient India has been differently evaluated by different writers. Some of those in favour of an early existence of the disease pointed to descriptions of a syndrome showing clinical features identical with those of true cholera in the ancient Indian medical literature particularly in the writings of Suśruta However Macnamara (1876) in his classical *History of Asiatic cholera* pointed out with much reason that

"Hippocrates, Galen and Wang-shooho have left us equally vivid accounts of this form of cholera in the various countries in which they lived But the more carefully we study the writings of these early authorities, the clearer it appears that they had never met with cholera in its epidemic or Asiatic form"

Sticker (1912) while sharing the misgivings expressed by Macnamara regarding possible references to true cholera in the classical Indian medical works, which were mute in regard to the epidemic prevalence of the choleraic disease they described drew attention to the following quotation taken by Schmidt (1950) from a Sanskrit work believed to have been written in Tibet during the reign of Ti song De tsen i e during the period from A.D 802 to A.D 845

"When the strength of virtues and merits decreases on earth, there appear amongst the people, first among those living on the shores of big rivers, various ailments which give no time for treatment, but prove fatal immediately after they appeared. At times the *nja* carries away the fourth part of the *dschambudwip* (?) it suddenly destroys the vigour of life and changes the warmth of the body into cold, but sometimes this changes back into heat. The various vessels secrete water so that the body becomes empty The disease is propagated by contact and infection. The *nja* kills invariably Its first signs are dizziness, a numb feeling in the head, then most violent purging and vomiting" [Trans.]

While certain that this was a description of true cholera, Sticker expressed doubts regarding the authenticity of the text—a point which it would be of great interest to settle

"so grievous was the throe and of so bad a sort that the very worst of poison seemed there to take effect, as proved by vomiting, with drought of water accompanying it as if the stomach were parched up and cramps that fixed in the sinews of the joints and of the flat of the foot with pain so extreme that the sufferer seemed at point of death the eyes dimmed to sense and the nails of the hands and feet black and arched"

Since Correa s time descriptions of cholera manifestations continued to be given by other Portuguese then by Dutch French and British obser vers, Macpherson in his *Annals of cholera* quoting 64 records by independent authorities referring to the presence of the disease from 1503 to 1817 ten of whom distinctly mentioned an epidemic spread of the manifestations they described It was inevitable that these reports were restricted at first to Goa the only province known to Europeans during the 16th century (Macnamara 1876) Afterwards however other areas on the west coast of India were mentioned successively Thus Thevenot (1689) who himself contracted the infection and Fryr (according to Macnamara the first Englishman who wrote about the disease) testified to the presence of cholera on the coast of Surat "some time prior to 1678" (Macnamara) As noted by Sticker (1912) Daman (Damão) near Bombay was affected in 1695

That the early records referred exclusively to the west coast of India appears to be due not merely to the circumstance that the British gained a foothold on the Coromandel coast and in Bengal in the east more than a century after the Portuguese had reached Goa Macnamara noted in this connexion that one of the earliest accounts of the occurrence of cholera in India from the pen of an English physician (Dr Paisley) and dated Madras (on the Coromandel coast) February 1774 was brought to light only 33 years afterwards, when it was printed in Curtis s work on the *Diseases of India* (Edinburgh 1807)—obviously because most of the early British observers insisted upon classifying the disease among the spasmodic affections instead of recognizing it as an affection *sui generis* and desig nating it Asiatic cholera. Therefore Macnamara concluded it was not surprising that no descriptions of this disease were given in the writings of British physicians even during the later part of the eighteenth and at the beginning of the nineteenth centuries Moreover as stated by this author

"our possessions in India prior to 1781 were surrounded by large provinces regarding whose habitants we had literally no knowledge whatever unto these territories the course of the epidemic could not possibly be traced"

It also deserves attention that the Hospital Board in Madras and Cal cutta was established only in the year 1786 so that before that year no regular reports on the incidence of cholera among the Europeans and the native soldiers were available

Nevertheless sufficient evidence exists to prove that during the last quarter of the eighteenth century cholera was not only met with on the east

However even if this reference should prove unreliable there is a second category of evidence which testifies to the early existence of cholera in India by showing that ancient religious rites were invoked to ward off the ravages of this disease

Macnamara stated in this connexion that the people in Lower Bengal had for a long time past worshipped the goddess of cholera it appearing,

"according to tradition, that, at an early period, the date of which cannot now be ascertained, a female while wandering about in the woods met with a large stone, the symbol of the goddess of cholera. The worship of the deity through this stone was, according to the prevailing ideas of the Hindoos, the only means of preservation from the influence of this terrible disease. The fame of the goddess spread and people flocked from all parts of the country to come and pray at her shrine in Calcutta "

As aptly pointed out by Macpherson (1872) whom Macnamara quoted the malady must have raged at times with violence or it would not have been found necessary to propitiate the deity specially on account of it.

Sticker maintained on the authority of Sanderson (1866) and of Tholozan (1868) that there was in a temple at Gujrat in western India a monolith dating back to the time of Alexander the Great, the inscription of which referred apparently to true cholera, saying

"The lips blue, the face haggard, the eyes hollow the stomach sunk in, the limbs contracted and crumpled as if by fire, those are the signs of the great illness which, invoked by a malediction of the priests, comes down to slay the braves " [Trans.]

While these statements strongly suggest that cholera has existed in India since immemorial times, irrefutable proof of its presence in historical times is furnished by the records of European observers who after the arrival of Vasco da Gama on the coast of Malabar in A.D 1498 had been given an opportunity to get acquainted with what was formerly a *terra incognita* to them. As emphasized by Macnamara,

"it is remarkable that in one of the very earliest communications of this description, written by a European, we have a clear and distinct reference made to Asiatic cholera, and this was the first account of the disease ever published. Doubtless, Asiatic cholera has flourished in the Delta of the Ganges, we know not for how long, but its ravages had not been witnessed by those capable of describing the disease "

This early record written by Gaspar Correa under the title *Lendas da India* (i.e *Legends of India*) referred to (*a*) a high mortality observed during the spring of the year 1503 in the army of the sovereign of Calicut, enhanced " by the current spring diseases, and smallpox besides which there was another disease sudden-like which struck with pain in the belly so that a man did not last out eight hours time ", and (*b*) an outbreak in the spring of 1543 of a disease called " moryxy " by the local people, the fatality rate of which was so high that it was difficult to bury the dead As described by Correa,

as cholera. There is little doubt that in the past this term has been used to cover a group of affections, such as *acute gastro-intestinal infections, colic, appendicitis, ptomaine poisoning*, etc., and cholera might have been mixed up with them. A significant point however is that no one, until at a late period, alludes to the epidemic character of the disease "

Nevertheless, Wong & Wu Lien teh did not believe that true cholera was entirely absent from ancient China stating that " one is perhaps justified in saying that it was present in this country in the 7th century "

Whether further importations of cholera into China took place before the nineteenth century seems uncertain Simmons in a report published in 1879 stated in this connexion that according to Cleyer an American author writing in 1873 the disease probably, imported from Malacca, appeared in China in 1669 and also claimed that Le Gentil (1779) in a work entitled *Voyages dans les mers de l'Inde* referred to an importation of cholera into China in the eighteenth century soon after the disease had been present on the Coromandel coast in 1761 and 1769 However while it is possible that Le Gentil made such a statement in one of his contributions to the *Mémoires de l'Académie Royale des Sciences* no reference to the spread of cholera from India to China could be found in his book, the two volumes of which appeared in Paris in 1779 and 1781 respectively

There can be little doubt that as Cleyer (quoted by Simmons, 1879) suggested in connexion with Malacca, early importations of cholera took place from India into neighbouring or not far distant countries, particularly into Burma However the only seventeenth century reference available in this respect deals with an appearance of the disease in Batavia Java, in 1629 observed by Bontius surgeon to the Dutch East India Company who recorded that the Governor General succumbed to the infection (Macnamara, 1876 Proust 1892) It was only during the last three decades of the eighteenth century when as noted already for the first time in its known history the infection showed a marked tendency to spread far afield that further information on an invasion of contiguous or neighbouring countries became available

To judge from the somewhat disjointed and certainly incomplete data assembled in regard to this period by Macnamara, in 1770 cholera was endemic in the Arcot region inland from Madras as well as throughout the Travancore area to the south west. From 1772 to 1782 the presence of epidemics was noted on the Coromandel coast. In March 1781 cholera was prevalent in the Ganjam district in the north-east of the province of Madras, and attacked within a few days 1143 men out of some 5000 Bengal troops marching through this area. According to a report on this visitation dispatched from Calcutta to the Court of Directors of the East India Company in London as quoted by Macnamara,

" the disease has not been confined to the country of Ganjam it afterwards found its way to this place [Calcutta] and *after chiefly affecting the native inhabitants*, so as

coast as well as in the west of India, but even spread beyond the confines of the sub-continent. However, before dealing with these developments it is necessary to devote attention to the question whether such a spread afield took place during previous times

General agreement exists that this question must be answered in the negative as far as Europe is concerned, even though a malady clinically identical with true cholera, and often designated by this name has been described by Hippocrates and many subsequent writers some of whom used other names for the ailment, e g. that of *weisse Ruhr*[1] It is true that this choleraic disease did not occur solely in sporadic form but that cases of this nature were not infrequently numerous and grouped together the appearance of this *forme catastique* of cholera being often ascribed to suitable atmospheric conditions (see for example, Fabre & Chailan, 1835) However even though the disease was apt to become prevalent at times it never showed a truly epidemic spread. This was emphasized by Macnamara, who referring to the manifestations of what Sydenham called cholera during the period 1679 82 in London stated that

"Sydenham makes no mention of a widely disseminated outbreak of the disease and Wells expressly states that the country was quite free from the malady and in fact one of its characteristic features was that its ravages were confined to the city of London"

Macnamara concluded, therefore, that the "cholera" manifestations observed by Sydenham and others stood in a relation to the true form of the disease similar to that between the bilious remittent fever of Bengal and the yellow fever of the West Indies. For

"the symptoms of a severe attack of bilious remittent fever are very similar to those present in cases of yellow fever nevertheless we cannot doubt that the two affections are produced by different causes, and that yellow fever is communicable whereas we are equally sure that bilious remittent fever is due to local influences and is certainly not transmissible by those affected with it to healthy people"

While these and many other observations[2] render it certain that no long-distance spread of cholera from India westwards took place before the nineteenth century this cannot be so confidently asserted in the case of China to the east.

It is true that, as Wong & Wu Lien teh (1934) aptly put it,

"the term huo luan the present name for cholera, is found in the Nei Ching and other old chronicles, but it appears that it does not refer to the disease we now recognize

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[1] As Haeser maintained, the etymology of the term cholera is uncertain. Celsus and others think it derived from χολή, the bile; Alexander Trallianus from χολάδες, the intestines. Kraus (*Kritisch-etymologisches medicinisches Lexicon*) and Littré (*Dictionnaire de médecine*) are in favour of the derivation from χολέρα, i e., the eaves (gutter). It speaks for this assumption that later Greek writers usually add the word νόσος (cholera morbus) However, modern writers seem in favour of the derivation from χολή, Macleod (1910), for instance, declaring that the Hippocratic term cholera originally meant bilious diarrhoea.

[2] It is significant, for instance, that the Arabian medical writers, when confronted by the 1821 cholera outbreak in Ocean (see page 19), had no name by which to designate the disease.

First Pandemic (1817)

Untenable though this contention is it must be admitted that in 1817 a new epoch in the history of cholera began because this year marks the onset of the first of a series of pandemics during which the infection after having gained impetus in India through a particularly severe and widely spread incidence extended its sway to other parts of the world paying heed neither to distances and natural obstacles nor to vain attempts at warding off its attacks through cordons and other quarantine measures One may claim therefore that cholera which as far as is known had hitherto been of more or less localized importance only began to become a most serious concern of the world in 1817 [1]

TABLE I CHOLERA PANDEMICS IN THE NINETEENTH CENTURY

According to					
Haeser (1882)		Hirsch (1883)		Sticker (1912)	
number	period	number	period	number	period
1 (a)	1816 23	1	1817 23	1	1817 38 *
(b)	1826-37		1826-37	—	— *
2	1840-50	3	1846-63	2	1840-64 *
3	1852-60	—	—	—	-
4	1863 73	4	1865-75	3	1863 75 *
				4	1881-96 *
				5	1899- *

* Kolle & Prigge (1928) stated that the 5th cholera pandemic (corresponding to Sticker's 4th) lasted from 1883 to 1896, and the 6th from 1902 to 1923.

It was probably not accidental that the onset of the first cholera pandemic fell within a period during which abnormal meteorological conditions prevailed In India, in particular the year 1815 and still more that of 1817 had been marked by extremely heavy rainfalls followed by disastrous floods and harvest failures while the year 1816 had been extraordinarily hot and dry (Sticker 1912) Whether *propter hoc* or *post hoc* it is certain that in 1817 cholera began to show an unusual violence in India. As claimed with much reason by Sticker this storm probably started in the hinterland of Bengal between the Ganges and Brahmaputra to reach Calcutta early in August i e before the presence of a "new" disease called "morbus oryzeus" as it was ascribed to the consumption of spoiled

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[1] As will be gathered from Table I above, which illustrates the views held by different writers regarding the dates of onset and duration of successive pandemics, Haeser places the beginning of the first of these in 1816 However, there is no convincing evidence in favour of this view which is not shared by other authorities.

to occasion a great mortality during the period of a fortnight, it is now generally abated and pursuing its course to the northwards "

As a consequence cholera broke out in April 1783 at Hardwar situated in the Uttar Pradesh (formerly the United Provinces) on the right bank of the Ganges, and apparently killed in less than eight days 20 000 of the pilgrims assembled at that holy place. At the same time the disease raged among the Mahratta armies engaged in war with Tippo Sultan

That this outbreak of cholera did not hold sway only in India is proved by reports quoted by Macnamara, which showed that (*a*) in March 1782 the disease was raging in epidemic form at Trincomalee in Ceylon severely affecting the British fleet at anchor in this port, which had probably suffered from cholera on a previous occasion already and that (*b*) during 1783 cholera existed in Burma.

Statements made to the effect that in 1775 cholera had reached Mauritius or as claimed by Fabre & Chailan, the nearby island of Réunion (then called Bourbon Island) are open to considerable doubt

Dealing with further developments, Macnamara summarized that in 1787 and again in 1794 cholera caused terrible ravages in Arcot and Vellore, while in 1790 it was once more prevalent in Ganjam Information on the years following, up to 1817 is scanty but, to judge from the occurrence of cholera cases among the European troops recorded by the Bengal Medical Board, cholera manifestations continued to occur in various parts of India, including, besides Bengal (where a violent outbreak appears to have taken place in 1814) also Bihar and Orissa, and the Madhya Pradesh (formerly the Central Provinces) as well as the Uttar Pradesh. Supplementing this information, Sticker besides referring to an outbreak at Travancore in 1792, also noted a further invasion of Ceylon in the year 1804

Incomplete or even fragmentary though the evidence brought forward above often is, it leaves no room for doubt that cholera, present in India since ancient times, not only continued to exist but was apt to manifest itself periodically in widespread conflagrations Further as aptly pointed out by Sticker even at this early stage one can clearly perceive the ominous role played in the propagation of the disease by military operations and by pilgrimages, when ample fuel became available for the spread of an infection either met with en route to the places of assembly or pre-existent there For the reasons adduced above it is not surprising, on the other hand, that the known early history of cholera in India furnishes hardly any clue for the cardinal epidemiological importance of Bengal which, according to the present state of our knowledge, has to be considered as the cradle, if not the original home, of the infection. However as will be discussed now observations made in that area from 1817 onwards filled this gap in the knowledge of cholera epidemiology in so dramatic a manner that some of the observers were led to believe that the disease had then arisen in Bengal *de novo*

"the great epidemic which had arisen in 1817, well nigh covering India within the three succeeding years had now subsided" In the meantime however cholera had become widely spread beyond the confines of the sub-continent

Bearing in mind that Burma and the island of Ceylon had suffered from cholera even in the past it is not surprising to find them involved in the widespread outbreaks starting in Bengal in 1817 As claimed by Sticker, Trincomalee was revisited by the disease in December 1818 but, according to Macnamara, the infection did not gain a foothold in Ceylon before 1819 when the ports of Jaffnapatam and Colombo became invaded From there cholera spread inland attacking not only the capital of Kandy but extending "well nigh over the length and breadth of the island"

To judge from scanty information Burma and possibly also Siam were invaded by the land route in 1819 (Hirsch, 1883) Bangkok the capital of the latter country became infected by the sea route in 1820 the whole country afterwards becoming devastated by the disease. Sea borne cholera broke out in Malacca in 1820 followed by epidemics in Penang and Singapore

As was inevitable the infection also spread to Java, Borneo and other islands of the Indonesian archipelago where it became manifest in 1820 or according to Hirsch (1883) even in 1819 The sufferings of Java were particularly great, 100 000 people succumbing on the island including 17 000 in Batavia alone While the Moluccas said to have been infected through ships from Calcutta were possibly invaded as late as 1823 cholera had already entered the Philippines in 1820 by way of Manila.

Dealing with the appearance of cholera in China, Wu Lien-teh (1934) maintained that the confines of the country had been reached by the land route as early as 1817 Be this as it may it is certain that the disease actually invaded China in 1820 via the sea route from Burma and Bangkok. After Canton had become first involved the infection also became manifest in the same year in the ports of Wenchow and Ningpo and spread into the Yangtze valley The north of the country became invaded in the following year Outbreaks in central and northern China, including Peking, recurred during the period 1822 24 It is of interest to add that, according to a statement made by Huc it is probable that cholera, proceeding from Peking crossed the Great Wall and followed the caravan route to Kyakhta thus reaching the Russian border

The disease made its first appearance in Japan in 1822, having been imported into Nagasaki by a merchant-ship from Java (Takano Ohtsubo & Inouye 1926) The infection rapidly extended to Osaka and some other cities, where it exacted a terrible toll in lives

The choler[illegible] [illegible]sion of Arabia taking place in the course of the first pandemic s[illegible] [illegible]ausal connexion with the landing of a British expeditionary fo[illegible] [illegible]y in 1821 from India to Oman The infection which

nce, had been reported on 23 August by Tytler the civil surgeon of Jessore, a town situated some 50 miles (80 km) north-east of Calcutta on a branch of the Ganges. That this was the real course of events is well shown by the reply to a report from Jessore given by the Calcutta Medical Board which, as quoted by Macnamara, stated in part

" that the disease is the usual epidemic of this part of the year It is understood that in certain quarters of Calcutta a similar epidemic prevails and it is probable that there is no considerable town in the low and humid climate of Bengal that is at present entirely free from its operation "

That the outbreak present at the time in Calcutta and soon officially designated " cholera morbus " nevertheless showed extraordinary features, is proved by a statement made on 17 September 1817 by the Calcutta magistrate wherein he said that the disease had

" of late been far more fatal than at any former period within the recollection of the oldest inhabitants, running its course generally in a few hours and sometimes in a few minutes "

The extraordinary virulence of the 1817 outburst is also well demon strated by the fact stated by Macnamara that

" within three months from its appearance the disease had been generated throughout *the Province of Bengal, including some 195,935 square miles [about 507 500 km²],* and within this vast area the inhabitants of hardly a single village or town had escaped its deadly influence "

The Bundelkhand, an area lying between what were later the United and Central Provinces and corresponding to present-day Vindhya Pradesh, was also overrun by the infection. The terrible toll which the disease exacted from the army of the Marquis of Hastings camping in that area is well illustrated by the following entry which, as quoted by Macnamara, the general made in his diary under 17 November

" The march was terrible for the number of poor creatures falling under the sudden attacks of this dreadful infliction, and from the quantities of bodies of those who died in waggons and were necessarily put out to make room for such as might be saved by the conveyance. It is ascertained that above 500 have died since sunset yesterday "

In 1818 cholera not only reappeared with undiminished violence in the places where it had raged previously but rapidly extended in various directions thus spreading north-eastwards into Nepal, directly or indirectly from the Bundelkhand over Agra and Delhi towards the Punjab which was eventually reached by the infection in 1820 as well as to Surat and to Bombay and in a southerly direction to Hyderabad, Bangalore, and Seringapatam Spreading from Ganjam, the infection also reached Madras and Madura.

While the disease continued to be active in 1819 and 1820 it tended to become localized in 1821 In the following year according to Macnamara

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The cholera invasion of Arabia taking place in the course of the first pandemic stands in causal connexion with the landing of a British expe ditionary force sent early in 1821 from India to Oman The infection which

first gained a foothold in Muscat, afterwards extended over the greater part of the territory and subsequently reached Bahrein to the west of the Persian Gulf as well as Bushire on its eastern shore, thus entering the territory of present-day Iran Spreading inland from there cholera successively invaded Shiraz and Teheran, finally reaching Resht, situated on the southern shore of the Caspian Sea.

As was inevitable, cholera also appeared in 1821 at Basra, the principal port at the head of the Persian Gulf and killed in less than three weeks between 15 000 and 18 000 people The infection was carried up the Tigris by boat and caravans and, reaching the region of Baghdad caused terrible havoc in the Persian army which attacked this city at the time. Subsiding during the winter cholera broke out once more in the spring of 1822 along the Euphrates as well as the Tigris. As vividly described by Macnamara, a Persian army which had defeated the Turks near Erivan and had pursued the enemy westwards, fell a prey to cholera. The victors retreated to Khoi in Iran where they dispersed disseminating the infection throughout the country As a result the disease spread northwards, reaching Tiflis (now Tbilissi) between the Caspian and Black Seas, and Astrakhan on the Caspian Sea which, however had been reached already by water borne infection from Resht. Whether these invasions took place in 1823 as stated by Haeser (1882) and Hirsch (1883) or in 1822 with recrudescences in 1823 as Macnamara (1876) seems to imply is difficult to decide

That the infection which had thus reached European territory did not become entrenched and progress farther was, in the opinion of Sticker due to the severe winter of 1823 24 rather than to the feeble control measures taken by the Russian authorities at Astrakhan. Sticker supported this view by pointing out that cholera also disappeared from the Tiflis area, where no preventive work had been done

Besides spreading in the manner described above cholera was also carried by caravans into Syria, reaching Aleppo in November 1822. It broke out in 1823 at Alexandretta (Iskenderun) and spread along the Syrian border of the Mediterranean, but entirely disappeared from this area by the end of the year

In addition to this more or less continuous spread, cholera made in the course of the first pandemic, two long-distance sprints

(*a*) The infection appeared at the end of October 1819 in Port Louis, Mauritius, evidently as the result of an importation by a ship from Trincomalee Ceylon, on which cholera has broken out en route Three weeks after the arrival of the vessel, which had landed some of her patients the disease became epidemic on shore and claimed over 6000 victims mostly Negro slaves. In spite of the precautions taken, the infection also invaded Bourbon Island (Réunion) where, however, only 187 casualties resulted.

(*b*) As recorded by Haeser (1882)

"in the course of its progress to Arabia, the epidemic [cholera] reached during the years 1820-21 also for the first time the near-by coast of Africa, but—to judge from very scanty information—spread only on the narrow coastal zone of Zanzibar (from the 4th degree northern latitude to the 6th degree southern latitude)" [Trans.]

This invasion, which was confirmed by Hirsch (1883) and by Clemow (1903) is not surprising in view of the dense traffic of Arabian dhows between Arabia and the East African coast—a route by which *Xenopsylla astia* was also carried to the latter area (Pollitzer 1954)

Summing up his description of the first cholera pandemic, Macnamara pointed out that

"the disease absolutely disappeared from Persia, Ceylon, Burmah and China after existing in these localities for three or four successive seasons—in fact, the epidemic cholera which had extended from India over these countries had again subsided into its endemic area in Lower Bengal—the Home of Cholera, as Dr Macpherson calls it"

Second Pandemic (1829)

Divergent opinions were held in the past regarding the origin of the second cholera pandemic It was believed in some quarters that it was due to a recrudescence of the infection which had persisted at Astrakhan since the time of the first pandemic However it would be impossible to reconcile with this assumption the fact that, before cholera became manifest at Astra khan in 1830 it had already appeared in 1829 at Orenburg (now Chkalov)

Dealing with the history of cholera in China, Wu Lien teh (1934) noted that in 1826 the infection was "again borne from India to China reaching Peking once more and steadily advancing, it crosses the Chinese wall, sweeps through Mongolia and eventually travels to Moscow"

However while this surmise might explain the appearance of cholera at Orenburg, it could not account for the second inroad of the infection to the west of the Caspian Sea. Little doubt can exist therefore, that, as advocated by Macnamara, the second as well as the first cholera pandemic can be traced back to Bengal where the infection had shown signs of increased violence and activity in 1826 This was followed still in the same year by a steady progress of the disease westwards along the Ganges and Jumna rivers and in 1827 by an invasion of the Punjab While informa tion for 1828 is indefinite it is known that in 1829 cholera was rampant in Afghanistan, penetrated into Persia, and was also present in the region of Bukhara and Chiva From there the infection was evidently carried by caravans to Orenburg in the south-east corner of European Russia, where an epidemic broke out at the end of August 1829 and from where cholera soon started to spread north westwards.

The infection seems to have subsided in Persia during the winter of 1829 30 but became active again in the spring of the latter year Spread ing northwards it once more reached Resht as well as Baku on the Caspian

Sea, and also reappeared at Tiflis and Astrakhan As maintained with much reason by Macnamara, it is probable that "the stream of cholera, which entered Russia from the northern provinces of Persia, formed a junction with that which flowed through Orenburg" What is certain is that cholera, which early in 1830 had come to a temporary halt in the Orenburg area, began in the spring an advance on a wide front which ultimately resulted in the invasion, not only of most parts of Europe but also of large parts of the Americas, as well as of Arabia and East and North Africa. The main features of this truly pandemic spread of the scourge which alone can receive attention within the scope of the present chapter will now be described.

Though every possible effort was made by the authorities to stem the tide with the aid of cordons and other rigid quarantine measures cholera steadily advanced into Russia, reaching Moscow by the autumn of 1830 There was a lull during the winter of 1830-31 but in the spring of the latter year cholera was again in full advance progressing (*a*) into the Baltic provinces and to St. Petersburg (now Leningrad) to spread from there into the north-western provinces of Russia as far as Archangel on the White Sea, as well as into Finland, and (*b*) into Poland, where the infection became entrenched among the Russian, and afterwards also among the Polish, troops at war in that country There can be no doubt that, as emphasized by Haeser (1882) and other authorities, the presence of cholera among these troops has to be considered one of the main causes for the further spread of the infection westwards. In fact, the situation in the Austrian province of Galicia became serious only after if had been entered by Polish and Russian contingents

From Galicia cholera passed into the interior of Austria Vienna becoming affected in August 1831 Before that time (in June 1831) Hungary had already been invaded, and here the disease raged with particular violence (Haeser) Outbreaks reappeared in Vienna and some other parts of Austria in 1832.

In spite of the most rigid quarantine measures it proved impossible to prevent the invasion of Prussia, the less so because *inter alia* the infection was carried by a ship from Riga to Danzig. Spreading into the interior of Prussia, the wave of infection reached Berlin in August 1831 while Hamburg became involved in October In several of the localities then affected in Prussia, including Berlin, and also in Hamburg cholera became recrudescent in the spring and summer of 1832. A limited outbreak commencing in August of that year in the Rhine province (Rhineland Palatinate) was evidently due to an importation of the infection from the Netherlands and not from the east.

The close shipping connexions existing between the Baltic and German ports on the one hand and England on the other made the importation of cholera into the latter country well nigh unavoidable. In fact the disease appeared in June 1831 on board some warships anchored in a creek of the Medway below London where vessels coming from Riga were in qua

rantine In October of the same year a cholera epidemic became manifest in the port of Sunderland on the east coast of England, but it could not be ascertained how or even when this outbreak had originated As noted by Macnamara the disease afterwards appeared at Newcastle, Gateshead, Edinburgh and in February 1832, at London the death toll in England amounting in November 1831 to 97 in December to 282, in January 1832 to 614 in February to 708 in March to 1519 and in April to 1401 Cholera recurred in England during the latter part of 1832 and visited before the end of August, Hull, York, Leeds and several other large towns The total number of cases in 1832 seems to have been 14 796, with 5432 deaths (Haeser Macnamara)

Cholera appeared in Dublin Ireland at the end of March 1832, and spread to many principal towns of that island

Considering that, until the end of 1831 cholera in Germany had been practically absent from the regions west of the Elbe river and that the out breaks in England had not assumed large proportions it is not surprising to find that France up to then remained free from the infection However in the middle of March 1832 the disease appeared in Calais and soon afterwards in Paris Cholera afterwards spread over the greater part of France, only 35 of the 86 departments remaining completely free mostly those in the southern and eastern mountainous areas.

Cholera appeared in Belgium in the spring of 1832 (first in a village near the French border) but claimed not more than 7984 victims. The disease seems to have caused also comparatively little havoc in the Nether lands where it first appeared at Scheveningen in June 1832

In the autumn of 1832 the presence of the infection was also recorded in Norway at Drammen, Moss, and Christiania Cholera was more widely spread in Norway during the following year but it was only in 1834 that severe epidemics took place (Hirsch 1883)

Besides showing a more or less contiguous spread in Europe cholera also reached in 1832, the distant shores of America it was first imported through the agency of ships from Europe which had been quarantined at Grosse Island a few miles below Quebec in Canada Cases appeared in Quebec early in June and during the following two weeks 1000 cholera deaths occurred in that city The disease spread with great rapidity along the St. Lawrence River and its tributaries into the interior

At about the same time the infection was also imported into the United States of America, where it appeared at New York on 23 June and at Philadelphia on 5 July Continuing to be rampant until 1834 cholera caused great ravages in the country even spreading, according to Haeser (1882) and Hirsch (1883) across the Rocky Mountains to the Pacific coast. A serious recrudescence of the infection in New York and other centres on the east coast in 1834 seems to have led to the invasion of Halifax in Canada

In the course of the second pandemic cholera also penetrated into other American countries. As claimed by Haeser it appeared as early as 1832 in Peru and Chile but the reliability of this information is denied by Hirsch Certain it is that in the spring of 1833 the infection became manifest in Mexico where the high plateau as well as the coastal areas became involved. In the same year cholera, apparently imported from Spain caused serious ravages in the island of Cuba. A recrudescence of the disease there in 1835 led to a further invasion of the USA where however besides New Orleans, the portal of entry only Charleston in South Carolina became affected (Hirsch)

While the appearance of the disease in the coastal areas of Guiana did not lead to serious consequences a devastating outbreak took place in 1837 in Nicaragua (Haeser) As added by Hirsch cholera appeared in the same year also in Guatemala

Though on the whole somewhat relenting in ferocity cholera continued to reappear in 1833 in some of the formerly affected European countries e g. in Hungary and even to spread to hitherto unaffected areas Thus the infection was imported early in the year into Portugal through a steamer which, carrying British troops had left England at the end of December 1832 and had had some cholera deaths en route Cholera, which broke out at the fort on the mouth of the Douro where the troops had been landed, soon spread, reaching Lisbon early in April 1833

In spite of quarantine measures enforced with truly Draconic severity in Spain cholera managed to penetrate into the country in August 1833 Remaining limited during this year the infection became widely spread in 1834 and even progressed at the end of the year into southern France (Marseilles and other places in Provence) Likewise the disease was carried from Spain to the opposite shore of Africa, particularly to Ceuta.

Another important event of the year 1834 was a serious visitation of Sweden which, as claimed by Haeser and Hirsch had hitherto remained free from cholera.

When dealing with the cholera manifestations in Europe during the earlier part of the second pandemic, it is not easy to decide how soon the north-eastern part of the Balkan peninsula (i e present-day Romania and Bulgaria) had become invaded. According to Macnamara an extension of the infection from southern Russia to these areas occurred as early as 1830, whereas Haeser and Hirsch recorded that they were invaded early in 1831 after the appearance of cholera in the Austrian province of Galicia Haeser added that at the end of July of that year an epidemic broke out at Constantinople (Istanbul) from where the infection was imported into Smyrna and other places in Asia Minor

Before dealing with the developments in Europe during the terminal years of the pandemic, attention has to be devoted to an ominous westward spread of the infection from Persia, the invasion of which in 1829 has been

noted above While Macnamara maintained that even before that time (? 1827) cholera had broken out among the troops of Said bin Sultan engaged in an attack on Bahrein according to Haeser it was only in 1830 that the infection progressed from Persia to Mesopotamia and Arabia, where plague was present at the same time In 1831 cholera which previously seemed to have been sporadic in Mecca broke out among the pilgrims assembled at this place killing nearly one half (? 12 000) of them

There can be little doubt that those of the pilgrims who were able to return to their homes in Syria Palestine and Egypt were responsible for the importation of cholera into these countries Appearing in Egypt first at Cairo (July 1831) cholera raged with the greatest violence penetrating up the Nile as far as Thebes as well as invading Alexandria and the whole delta of the Nile Returning pilgrims were probably also instrumental in carrying the infection to Tunisia where cholera broke out soon after it had appeared in Egypt

While cholera seemed to show signs of a decline in Europe during the year 1834 in 1835 it again became rampant in several parts of the continent As noted already the infection had been carried at the end of 1834 into Provence The resulting epidemic in Marseilles on 7 December terminated at the end of March 1835 However in June a second and far more violent outbreak commenced at the acme of which (24-26 July) 1500 persons succumbed The disease also raged at Toulon and many other places in southern France

Before dealing with the most serious consequences of this recrudescence of cholera for other parts of Europe it should be mentioned that at the end of 1834 and much more markedly in 1835 cholera became manifest among French troops sent to Algeria The civilian population became involved and the infection penetrated deep into the hinterland According to Hirsch cholera was again " disastrously prevalent " in Algeria in 1837

During the period of 1835 37 cholera also displayed great activity in Egypt and appeared in Tripolitania and Tunisia as well as south of Egypt in the Sudan and Abyssinia The disease also reappeared in 1836-37 on the Somali coast and in Zanzibar

Considering that (*a*) cholera raged with great ferocity on the Malabar coast of India in 1833-34 and (*b*) the disease was present in epidemic form at Mecca during the 1835 pilgrimage Macnamara postulated with much reason that these cholera manifestations in north-eastern and East Africa were due to a fresh importation of the infection from India He even claimed that the same held true in regard to the developments in Europe during the period of 1835-37 but one must agree with Haeser that enough remnants of the infection had been left in that continent to account for the recrudescence or spread of cholera.

It should be noted in this connexion that the infection progressed through the Riviera from France into Italy and spread in the latter country

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Before dealing with the developments in Europe during the terminal years of the pandemic attention has to be devoted to an ominous westward spread of the infection from Persia the invasion of which in 1829 has been

Progressing along the trade route from Canton to Burma the infection permeated into the northern part of the latter country in 1842 and branched southwards along the Irrawaddy River towards Rangoon That at the same time cholera inexorably pursued its westwards course is convincingly shown by the statement of an envoy from Sinkiang (Chinese Turkestan) who told Macnamara that in the year 1844 a malady of the nature of cholera

"came from the side of China that during that summer it attacked all the places on or near the main line of traffic from China that in Kashgar Yarkund, Kokand and Bokhara, it killed thousands of people that it lasted for a few weeks in each place and the people died by hundreds every day "

Thus cholera had progressed once more into the area of Bukhara which, as noted before, had been invaded early in the second pandemic. However while in 1829 the invasion of this area was due to a direct spread of the infection from India, in 1844 cholera though originally derived from Bengal had arrived in the Bukhara area by a long indirect route More curious still the evidence assembled by Macnamara leaves no room for doubt that, just as it had made earlier in its course a sidetrack into Burma so cholera, as soon as it met with other paths leading southwards, penetrated into Afghanistan (where it reached Kabul in 1844) and then into the Punjab from where it extended in 1845 south westwards to Karachi and south-eastwards to Delhi

As stated by Macnamara cholera, continuing at the same time to follow its main course

"spread as far west as the town of Meshed before the close of the year 1845 and it burst forth there again with renewed violence in the June of the following year quickly extending to Teheran and Tabreez, and overspreading the province of Ghilan before the close of the year it reached as far north as the town of Derbent on the Caspian Sea"

The south-eastern corner of Europe had thus been reached by the pandemic wave The infection does not seem to have progressed beyond Derbent, a Caspian port north of Baku during the winter 1846-47 Presumably however in the latter year new impetus was given to it through the developments described below which resulted in a second cholera invasion of Persia

A serious recrudescence of cholera in Lower Bengal in 1845 had led in the course of the same and the following years to an invasion of Madras and Ceylon on the one hand, and of the Bombay area on the other Progressing westwards from there "in the month of May 1846 cholera showed itself at Aden, Mocha and Jeddah and invaded almost the whole of the sea board of the Arabian peninsula it even penetrated into the interior of Omaun" (Rigler quoted by Macnamara)

There can be little doubt that this spread of the infection in Arabia led to a cholera invasion of Persia the less so as it is definitely known

from 1835 to 1837 At the end of this period (1837) the disease appeared also in the Maltese islands From upper Italy cholera penetrated in 1836 into the Tessin canton of Switzerland and into the Tyrol A few places in Istria, Croatia, Dalmatia, Carnolia, and Styria also became affected at the same time

A serious epidemic recurred in Vienna and cholera spread from there into the northern parts of the Austrian Empire and also into Hungary

From Tyrol the infection penetrated into Bavaria reaching Munich in October 1836 In the same year there occurred an outbreak at Coventry in England, and cases on a warship anchored near Greenwich.

In the summer of 1837 there were recurrences of cholera in Prussia, Hamburg, and Poland In the following year no more epidemics developed in Europe, but here and there sporadic cases still occurred

Information regarding the inroads of cholera into the countries east of India during the second pandemic is scanty Haeser remarked in this connexion that the infection which had been introduced during the first pandemic into the Dutch East Indies (now Indonesia) and the Philippines, persisted there until 1830 and also claimed that in 1832 cholera reached the Swan River region of Australia, but showed no tendency to spread there. In the opinion of Hirsch, however " the statement that cholera prevailed on the west coast of Australia (*Gaz méd de Paris* 1832, p 499) rests upon hardly reliable newspaper information "

The Straits Settlements suffered from epidemic cholera in 1826 but then remained free until 1840 As noted before, cholera was reintroduced into China in 1826 In the following year the disease was said to be present in Chinese Tartary, while in 1835 an outbreak (presumably due to a recent introduction from India) was recorded at Canton. According to Hirsch cholera reappeared in Japan in 1831

While fairly quiescent in India during the years 1835 and 1836 cholera became prevalent in Lower Bengal in 1837 and then spread westwards as far as Afghanistan where an outbreak in Kabul in 1839 was recorded.

Cholera became rampant once more in Lower Bengal early in 1840 at a time when a large number of troops had been assembled in Calcutta and Madras to embark for active service in China. No doubt can exist that the contingents from Calcutta were responsible for importations of the infection first into the Straits Settlements and then into China, where an initial epidemic broke out soon after landings had been effected on the island of Chushan outside Shanghai in July 1840 The infection soon spread to the mainland where it persisted for this and the following two years, inflicting, as Macnamara put it, " on the unfortunate inhabitants of the Celestial Empire one of the most frightful visitations of disease to which any nation was ever subjected "

Besides extending eastwards into the Philippines, cholera, spreading westwards from Canton started on a long journey in the course of which many countries were to be devastated

Progressing along the trade route from Canton to Burma the infection permeated into the northern part of the latter country in 1842 and branched southwards along the Irrawaddy River towards Rangoon That at the same time cholera inexorably pursued its westwards course is convincingly shown by the statement of an envoy from Sinkiang (Chinese Turkestan) who told Macnamara that in the year 1844 a malady of the nature of cholera

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As stated by Macnamara, cholera, continuing at the same time to follow its main course,

"spread as far west as the town of Meshed before the close of the year 1845 and it burst forth there again with renewed violence in the June of the following year quickly extending to Teheran and Tabreez, and overspreading the province of Ghilan before the close of the year it reached as far north as the town of Derbent on the Caspian Sea "

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that the disease had gained an entry into Mesopotamia, reaching Baghdad in September 1846 and then spreading northwards up the Euphrates and Tigris

As noted above it was probably due to the added effect of this second invasion of Persia that cholera, which had become latent at Derbent during the winter of 1846-47 not only reappeared in this port in April 1847 and spread along the Caspian shore to Astrakhan and then up the Volga, but also broke out in July at Tiflis and progressed from there westwards to the Black Sea coast and north westwards across the Caucasus mountains into the interior of Russia Moreover progressing possibly up the Ural River the infection reached the Orenburg area and from there spread rapidly into Siberia to reach Tobolsk "previous to July" (Hirsch)

Before dealing with the further advances of cholera in Europe and subsequently also America attention must be devoted to a second ominous inroad of the infection farther southwards, which culminated in an epidemic killing more than 15 000 people at and near Mecca in November 1846 the disease having been imported probably from the port of Jidda on the Red Sea and not overland from the east.

The progress of cholera resulting from the above-described invasion of Russia was rapid during the summer of 1847 Moscow being reached in September Soon afterwards derived probably from the Black Sea ports the infection became manifest in Constantinople However as was usual even during the periods of the most active spread of cholera, there was a lull during the winter of 1847-48 when according to Macnamara, Olgopol (a place about 30 miles (48 km) east of the Austrian frontier) and the vicinity of Riga had been reached

Resuming its march early in 1848 cholera progressed not only in Europe reaching Norway in the north, the Balkan countries in the south, England, Scotland, and Ireland in the north west, and Spain in the south west, but was carried on the one hand to Egypt by way of pilgrims returning from Mecca, and on the other to the USA, reaching Staten Island outside New York, and New Orleans, and continuing to spread—still in the same year—from the latter port far up the Mississippi and also to Texas Thus, as stated by Macnamara, "between May and December 1848 cholera had extended its influence from Moscow (37°E longitude) to the southern part of the United States of America (90°W longitude)" Moreover a reappearance of cholera at Constantinople led to the invasion of Asia Minor Syria, Palestine and possibly even Persia (Haeser)

Following a comparatively quiet spell during the winter cholera reappeared in the spring of 1849 over the greater part of Europe The whole of France became involved, the infection spreading from there into Italy as well as to North Africa (Algeria and Tunisia) The ravages of the disease in England were pathetically described by Farr (1852) thus

"If a foreign army had landed on the coast of England, seized all the seaports, sent detachments over the surrounding districts, ravaged the population through the summer

after having destroyed more than a thousand lives a day for several days in succession and in the year it held possession of the country slain 53,293 men women and children the task of registering the dead would be inexpressibly painful and the pain is not greatly diminished by the circumstance that in the calamity to be described the minister of destruction was a pestilence that spread over the face of the Island, and found in so many cities quick poisonous matters ready at hand to destroy the inhabitants "

Justifying the designation of "America s greatest scourge" given to it by Chambers (1938) cholera also caused widespread ravages in 1849 in the USA where—owing to the appearance of an epidemic in May of that year—New York City had become a most potent centre for the distribution of the infection Spreading from there and also continuing its progress from New Orleans cholera overran practically the whole of the States lying east of the Rocky Mountains and made inroads into Canada which however was also invaded by the sea route directly from Europe Moreover the infection spread by various routes into Mexico and was also carried at the end of 1849 by ship from New Orleans to the river Chagres in Panama

During the year 1850 cholera reappeared in a virulent form in Egypt, and spread from there along the whole coastal area of North Africa In Europe it was reproduced in most areas which had been visited in 1849 and appeared *de novo* in Denmark and Sweden in the north, and in the Maltese and Ionian islands in the south The mainland of Greece was spared on this occasion was well as in 1832 and 1837

Extensions of the infected areas also took place during 1850 in the Americas California was reached by ship from Panama to San Francisco and by the overland route to Sacramento In South America cholera penetrated into Colombia as far up as the plateau of Bogotá and—to judge from somewhat unreliable accounts—also into Ecuador to become prevalent at Quito (Hirsch)

Besides being prevalent on the American continent cholera raged in 1850 and again in 1851 with rarely paralleled violence in Cuba and in Jamaica, which then seems to have been visited for the first time From Cuba the infection was carried in May 1851 to Grand Canary Island, where it caused no less than 9000 deaths, most of them within the space of a few days

In North Africa in 1851 cholera was a serious menace only in Morocco Outbreaks in Europe during that year were restricted to Poland Silesia and Pomerania while elsewhere the pandemic seemed to have subsided Noting, however that in 1852 the disease not only reappeared in Poland but spread from there into some of the adjacent provinces of Russia as well as into Prussia some writers such as Tholozan (1868) and Hirsch incriminated a persistence of the infection in Poland as the cause of the new pandemic spread of cholera commencing in 1852. Still while it would be wrong to disregard the merits of this contention there can be no doubt

that much impetus was added to this renewed activity of cholera through a fresh wave of infection starting in India in 1849 The result was that, according to Macnamara,

"at the end of 1852, the inhabitants of the northern and western provinces of Russia were under the influence of the cholera of 1848-49 and the inhabitants of her Caucasian provinces were again subjected to a fresh importation of the disease from western India through Persia"

Third Pandemic (1852)

There can be no doubt that during its course as well as at its commencement the third cholera pandemic was the combined result of local recrudescences due to a temporary entrenchment of the infection and of repeated importations of the disease so that, as noted by Macnamara, it was no more possible to trace its course step by step as could be done in the previous outbreaks

The main features of the third cholera pandemic from 1853 onwards may be described as follows

Besides raging in Persia and Mesopotamia, as a consequence of an 1852 outburst in India, cholera was rampant in 1853 in the northern part of Europe and also reached the USA Mexico and the West Indies

In 1854 the infection continued to exact a serious toll in some countries of northern Europe for example England but was particularly rampant on the continent in the south The transport of troops from southern France effected on account of the Crimean War was no doubt responsible for the appearance of cholera in Greece and Turkey In the west the disease not only raged in most parts of the USA and Mexico and in some of the West Indian islands but also appeared in Canada and in Colombia on the northern shore of South America The only consoling feature amidst the calamities caused by the infection in 1854 one of the worst cholera years on record was that observations made in England clearly showed, to those who were not obsessed by fanciful theories that contaminated water played a major role in the spread of cholera and that consequently a supply of safe drinking-water was of cardinal importance in the prevention of the disease.

Besides reappearing in 1855 in many of the areas affected during the previous year cholera, which had probably gained impetus through a most serious recrudescence in India, appeared in countries hitherto not, or not seriously affected during the pandemic. In the Near East the infection spread via Arabia into Syria and Asia Minor In Africa the disease appeared in Egypt, spread into the Sudan and along the north coast as far as Morocco and also visited, for the first time the Cape Verde islands. In Europe the infection penetrated into previously unaffected parts of Italy and adjacent parts of Austria and made an inroad into Switzerland North America was apparently free but cholera broke out in Venezuela and Brazil.

Except in Spain and Portugal (including Madeira) cholera did not cause much havoc in Europe during the period 1856-58 However the disease was rampant during these years in India, where spread of the infection was fomented by the disturbances of the mutiny and the subsequent military operations.

Cholera which commencing an eastward spread early in the pandemic had reached Indonesia in 1852 and China and Japan two years later became most serious in these two empires during the period 1857 59 The Philippines were revisited in 1858 while Korea suffered from the disease in the following year

Other noteworthy events of the period now under review were (1) four outbreaks of cholera from 1854 to 1862 in Mauritius and one (1859) in Réunion and (2) serious inroads of the infection into East Africa where Zanzibar serving as the main distributing centre the infection spread along the coast to Mozambique in the south and from there to Madagascar and the Comoro Islands as well as inland into Uganda. As added by Haeser and Hirsch cholera which had already invaded Abyssinia (Ethiopia) in 1853 reappeared there in 1855 and more markedly in 1858

In the Americas cholera manifestations were recorded in 1856 in various parts of Central America and during that and the following year also in Guiana

In 1859 cholera showed signs of a much increased activity ushered in by a serious recrudescence of the infection in Bengal From India the disease spread following its old routes, westwards into Persia Mesopotamia, and Arabia and in a north western direction into Russia It is uncertain however to what extent the outbreaks subsequently taking place in that country as well as in other parts of Europe (Sweden Denmark, Mecklenburg Schwerin western Prussia, the Netherlands and Spain) were due to this fresh importation or to local reactivation of latent infections Probably being imported from Spain the infection appeared in 1859 also in some ports of Morocco and Algeria.

Apart from a serious recrudescence in Spain in 1860 in the course of which Gibraltar became involved, and slight cholera manifestations in St. Petersburg, where the infection seems to have lingered on until 1864 Europe seems to have become free from cholera at the end of 1859

Fourth Pandemic (1863)

The fourth pandemic beginning in 1863 and lasting according to Haeser until 1873 or as maintained perhaps more appropriately by Hirsch and Sticker until 1875 stood in marked contrast to the previous pandemics because as summarized by Haeser

"cholera did not penetrate into the heart of Europe as previously over its ancient paths through Persia, the Caspian sea ports, etc., but by new traffic routes which had been created

that much impetus was added to this renewed activity of cholera through a fresh wave of infection starting in India in 1849 The result was that, according to Macnamara,

"at the end of 1852, the inhabitants of the northern and western provinces of Russia were under the influence of the cholera of 1848-49 and the inhabitants of her Caucasian provinces were again subjected to a fresh importation of the disease from western India through Persia"

Third Pandemic (1852)

There can be no doubt that during its course as well as at its commencement the third cholera pandemic was the combined result of local recrudescences due to a temporary entrenchment of the infection and of repeated importations of the disease so that, as noted by Macnamara, it was no more possible to trace its course step by step as could be done in the previous outbreaks.

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Spain, infected in July 1865 by a traveller arriving in Valencia from Alexandria via Marseilles, suffered appreciably, but the disease became sporadic in 1866 and then disappeared An extension of the infection from Spain into Portugal led to outbreaks only in a few places Cholera also did not assume serious proportions in 1865 in England An invasion of Luxembourg in the same year was of importance in so far as an exacer bation of the situation there in 1866 led to an appearance of cholera out breaks in the Rhineland Palatinate and Westphalia in 1866 and 1867

Curiously the infection also appeared in the autumn of 1865 in Saxony having been imported by a woman who arrived in Altenburg with her cholera affected child from Odessa and soon fell a victim to the disease 468 cases resulted

While cholera showed but little activity during the winter of 1865-66 the infection flared up once more in the spring of the latter year thus ushering in a season which Haeser considered one of the most distressing episodes in the history of epidemics How far the ravages then caused by cholera in Europe were due to renewed importations of the infection from the east and not to local recrudescences is difficult to decide, the more so as the data supplied regarding this question by Haeser and by Macnamara respectively show a marked discrepancy No doubt can exist that the war waged by Prussia against Austria and her allies as well as the hostilities between Austria and Italy exerted a most unfavourable influence on the cholera situation in central Europe

In Russia cholera extended its sway from the Caucasus as far as St. Petersburg and from Orenburg to the western border of Poland, claiming in 1866 a toll of more than 90 000 lives The disease reappeared in the spring of 1867 but caused much less havoc

With the exception of Sweden, which recorded 4503 cholera deaths, the Scandinavian countries suffered little in 1866 In Germany, on the contrary epidemics breaking out in several regions caused a great loss in lives the cholera deaths in Prussia alone amounting to almost 115 000 The situation was also most serious in war torn Austria Hungary result ing in a cholera mortality of about 80 000 in Bohemia and Moravia while other parts of Austria also suffered and 30 000 succumbed to the disease in Hungary In Italy there was a serious cholera recrudescence in 1866 for which the military operations were largely responsible During that year cholera also led to almost 20 000 deaths in the Netherlands and over 30 000 in Belgium. In Great Britain cholera became manifest in many places but usually did not spread so that the death toll from the disease totalled not more than 14 378 5596 succumbing in London, 2501 in Ireland, and 1170 in Scotland.

Generally speaking, cholera was far less severe in Europe in 1867 than during the previous year An exception was formed in Italy where wide spread epidemics involving even Sardinia, led to 130 000 deaths Importa

n the meanwhile over Arabia into Egypt, Constantinople, southern France and Italy" [Trans.]

Opinions as to how and when Mecca was reached by cholera from India during the initial stage of the pandemic were at variance It was claimed that the disease had been brought to Arabia by pilgrims reaching Jidda by ship from India and even Malacca, but Macnamara, while not denying that this might have been the case declared that

"to attach undue importance to such incidents to the neglect of those broader features presented by the disease in its course from Bengal into Arabia and the Hadjiz, is to complicate the subject, and tends to withdraw our attention from the major to minor details in the history of this remarkable epidemic"

Whether cholera was already present in Mecca at the time or was imported in 1865 only it is certain that conditions for a rapid spread of the infection were particularly favourable in that jubilee year when extra ordinarily large numbers of pilgrims were assembled. The outbreak taking place in May 1865 was, therefore of extreme violence Macnamara stating that probably including those who succumbed at Jidda, not less than one third of the 90 000 pilgrims assembled at and near Mecca fell victims to the disease.

The infection was carried from Mecca by returning pilgrims to other parts of Arabia, Mesopotamia, Syria, and Palestine as well as—most fatefully— by the sea route to Suez which was by then connected with Alexandria by a railway As a consequence cholera broke out in the latter city at the end of May or early in June Though the epidemic ensuing there was not particularly severe Alexandria became a distributing centre from where the infection was carried by refugees into other parts of Egypt and by steamer to several Mediterranean ports among which Istanbul, Smyrna, Ancona, and Marseilles became the most important subsidiary distributing centres.

From Istanbul, which had already been reached in July 1865 the infection spread over Turkey as well as southwards to Asia Minor Cyprus, Rhodes and some of the Ionian islands, and north-westwards into Bulgaria, Romania, and apparently also into the (then) Austrian province of Bukovina.

Russia was invaded by different routes from the south but nevertheless suffered little in 1865 and early in 1866 the infection remaining restricted to six governments.

However having entered through Ancona, cholera became serious in southern Italy including Sicily The infection also became fairly wide spread in France, where Paris became affected in September 1865 but there were only about 10 000 victims in the whole of the country Persisting through the winter cholera reappeared in 1866 in many parts of France In 1867 only a few of the formerly affected districts suffered to a slight extent.

Whether cholera reached the USA in 1865 or in 1866 is uncertain Chambers considered it possible that the infection, imported by several ships from Le Havre appeared at New York in the autumn of the former year, but was soon suppressed by the cold weather The onset of a serious outbreak in May 1866 might, therefore, have been the result of a recrudescence of the infection and not of its recent importation by cholera affected ships particularly the German steamer *England*, as assumed by Haeser It is certain that cholera was rampant in New York during the summer and autumn of 1866, the official figure of about 2000 deaths being probably far below the mark

The further spread of the infection in the USA was facilitated by a considerable extension of the railways into the interior of the country, which had taken place since 1849 An even more ominous role in the spread of cholera there during 1866 was played by troop movements due to the reorganization of the army after the war between the States. Military encampments like that at Newport, Kentucky, thus became subsidiary distributing centres of the infection, in addition to several of the major cities such as New Orleans, where the disease, probably imported by troopships from New York, appeared in July and, lasting until October, claimed a toll of about 1200 lives

In contrast to previous outbreaks the role of New Orleans as a distributing centre was limited because as aptly stated by Chambers,

"trains from the Eastern ports outstripped the steamboats to Cincinnati, Louisville, Chicago and St. Louis in carrying the seeds of the scourge, just as they were winning the race for the commerce, travel and romance of the upper interior valley"

However transport of the infection by ships, particularly by vessels carrying troops, was responsible for the appearance of cholera in several localities of Louisiana as well as of other southern States, including Texas

The spread of the disease by the railway traffic was responsible for the appearance of cholera in the Middle West as far as Kansas A solitary infection observed at Albuquerque New Mexico indicated according to Chambers, the western limit of the 1866 invasion

Though, as estimated by this writer the number of cholera deaths occurring in the USA during 1866 possibly amounted to 50 000 it deserves attention that, according to him

"even so the mortality in 66 did not compare to that of previous epidemics. While estimates for the whole country were not even attempted for either of the previous epidemics, in 33 a mortality of 5 percent, 10 percent or even 15 percent of the population of a locality was not unusual the mortality in 49 seldom reached 10 percent while in 66 we know of no considerable community where the mortality reached 5 percent"

As was to be expected in 1867 a recrudescence of the infection was observed in many of the principal cities which had suffered from cholera during the previous year With few exceptions, however, these manifestations were restricted to a few or a limited number of cases A major outbreak took place at New Orleans, which suffered at the same time from

tions of the infection from Italy led to sporadic attacks or limited out breaks in Switzerland. In 1868 cholera reappeared in only a few European localities particularly in Essen North-Rhine, Germany and in Reggio di Calabria and Messina, Italy

Besides raging in Europe and, as will be discussed below, in the Americas, during the period now under review cholera showed an amazingly extensive spread in Africa.

An importation of the infection, apparently from Bombay via Aden, taking place in 1864 led to an invasion of Somaliland, where cholera caused great ravages in 1865

In February 1865 the infection was carried across the Red Sea from Jidda to Suakin and Massawa and penetrated from there into Abyssinia (Ethiopia) Continuing a southward course, cholera eventually (1869) reached the region of the Kilimanjaro and spread from there in various directions particularly (*a*) south westwards to and across Lake Tanganyika to invade finally in 1870 the upper reaches of the Congo River and (*b*) south-eastwards to Zanzibar island where in 1869 70 000 persons suc cumbed to the disease

Progressing also from the south end of Lake Tanganyika along trade routes on the western shore of Lake Nyasa, cholera reached, in May 1870 the city of Mozambique This port, like Zanzibar became a distributing centre of the infection which was thus carried to the Comoro Islands, Madagascar and the Seychelles

The countries on the Mediterranean shore of Africa, which were also ravaged by cholera during the period under review seem to have been invaded by various routes. Thus it was claimed that in 1867 cholera was imported into Tunisia by smugglers from Sicily while the infection of Algeria in 1865 was probably derived from France. The Algerian invasion culminated in an outbreak taking place in 1867 and was alleged to have caused 80 000 deaths.

Similarly Morocco though already infected through pilgrims returning from Mecca in 1865 had its most violent outbreak in 1868 when the disease, imported from Algeria, seems to have progressed from the hinterland towards the coast.

In 1868 cholera, carried probably by caravans from Morocco appeared at Podor on the Senegal River in French West Africa and then progressed to St. Louis. From there the infection spread, via MacCarthy Island, to Bathurst in Gambia and Bissau in Portuguese Guinea (1869) According to Macnamara, at Bathurst cholera carried off 1700 victims out of a popula tion of about 5000

During the period 1865-70 cholera became epidemic in several West Indian islands—first, imported from Marseilles, in Guadeloupe, where it claimed in 1865-66 almost 12 000 victims among a population of about 150 000 then in Santo Domingo (1866) St. Thomas (1868) and Cuba (1867 70)

Westwards, cholera spread in 1871 from Russia to (*a*) Finland and Sweden, where no major epidemics took place (*b*) Prussia, and (*c*) the Austrian province of Galicia

The infection spread in Prussia during the summer of 1871 as far as Berlin and also reached Hamburg but except in East Prussia no major outbreaks resulted While during the year 1872 cholera remained sporadic in the easternmost part of Prussia, major outbreaks causing a total death toll of 33 156, took place in 1873 in many parts of Germany including, besides Prussia and Hamburg, Bavaria, Württemberg Baden, and Hesse. During the winter of 1873-74 cholera remained manifest in Bavaria (particularly in Munich) and in a district of Prussian Silesia, where a major outbreak occurred in the spring of 1874

Austria had serious outbreaks in 1872 and to a much lesser extent, in 1873 Hungary suffered severely during these two years, when cholera claimed a total of 190 000 victims

Though repeated importations of the infection into Great Britain took place during the period under review it was invariably possible to prevent a spread of the infection Similarly, the appearance of sporadic cases in the Netherlands and Belgium did not lead to serious consequences Slight outbreaks were noted in 1873 in Sweden and at Bergen in Norway In France cholera appeared at Paris as well as in several other districts, a major epidemic developing at Caen.

In the USA, New Orleans and the Mississippi basin once more became seriously involved during the year 1873

Besides India where cholera raged with particular violence in 1875 (364 755 deaths) other Eastern territories suffered severely during the concluding years of the pandemic

An exacerbation of the cholera situation in Persia where, as noted above, the infection had become entrenched since 1865 led to most violent outbreaks in 1870 and to a spread of the infection into Turkish Kurdistan Mesopotamia, and Arabia.

During 1871 72 the infection, derived possibly from Persia besides progressing westwards to Egypt, spread in an eastern direction into Bukhara and Russian Turkestan

A reappearance of cholera at Mecca in 1872 resulted in an invasion of cholera via Suakin into the Sudan

It also deserves mention that in 1875 Syria was devastated by a cholera outbreak of unknown origin.

To judge from scanty information the regions in Asia to the south-east and east of India repeatedly suffered from cholera throughout the pandemic now under review As stated by Wu Lien teh, in 1862 the disease was widespread in China, reaching Peking and Manchuria. Thousands of people were stated to have fallen victims to the infection in Shanghai

yellow fever While the latter disease claimed over 3000 lives, the number of cholera cases was restricted to 575 Some spread of cholera from New Orleans to adjacent territories took place, apparently brought about mainly by troop movements.

While Canada remained almost free from the infection during the period under review an importation of the disease from New Orleans led to cholera manifestations in Central America (Nicaragua and British Hon duras) from 1866 to 1868 At the same time the disease first becoming entrenched among Paraguayan troops engaged in war against combined forces of Argentina and Brazil in April 1866 reached, in the autumn of that year the Argentinian city of Corrientes. A recrudescence of the infection there early in 1867 led to a spread of cholera down the Paraná River in the course of which Buenos Aires was reached in December In 1868 Uruguay also became affected Involvement of the interior provinces of Argentina in 1869 led to an overland invasion of Bolivia and Peru, where the disease spread from the hinterland to the coast. As maintained by Hirsch, in contrast to Haeser this was the first appearance of cholera on the west coast of South America.

In addition to the above mentioned countries, Brazil became invaded by cholera in April 1867 Entering from Paraguay the infection spread in the States of Rio de Janeiro and Rio Grande do Sul and again became prevalent in 1868

While, as noted above, in 1868 cholera became manifest in only a few places in central Europe and the west of the continent remained free, the infection continued to persist during that and the following year in Russia, but did not, as a rule cause much havoc A moderately severe epidemic taking place at Kiev in August 1869 was, in the opinion of Macnamara, possibly the result of a reimportation of the infection from Persia, where cholera raged perennially from 1865 to 1871 It is noteworthy however that a minor outbreak had already taken place in Kiev in 1868

Cholera was more active in Russia during 1870 when 37 governments suffered. In the following year the disease raged in practically all parts of European Russia as well as in the Tobolsk and Tomsk governments of Siberia, claiming a total death toll of 130 000 Almost the same mortality was recorded in 1872, when the southern and western governments in particular were involved. In 1873 there were but few outbreaks in Russia proper but cholera remained active in Poland during that and the following year

During 1871 cholera spread from Russia in various directions. Southwards the infection was carried to Black Sea ports in Romania and Bul garia and also to Istanbul and Trabzon in Asia Minor Manifestations of the disease in other localities of Asia Minor in 1871 and 1872 probably stood in causal connexion with these invasions Cholera also became prevalent in Romania in 1872 and, more markedly in 1873 when the infection spread into Bulgaria and from there to a slight extent also to Salonica.

the arrival of an infected sailing vessel, took place on the island of Yeu in the Bay of Biscay (in der Beeck 1948)

Though an attempt was made to protect Italy through quarantine measures, cholera became widely spread there in 1884, but caused great havoc only at Naples where, in August and September, over 10 000 cases and more than 5000 deaths were recorded The infection persisted in Italy and again became widespread in 1886 and 1887, but no further major epidemics developed.

Spain did not suffer severely from cholera in 1884 (592 deaths), but in the summer of 1885, when the provinces of Valencia and Murcia in particular became afflicted, the case incidence rose to 160 000 with almost 60 000 deaths The country was once more visited by cholera in 1890

Though cases were repeatedly imported into Great Britain the infection invariably failed to entrench itself both because adequate measures were taken and because wholesome water supplies (*eine für alle Zwecke der Reinlichkeit genügende Wasserversorgung* (M Pettenkofer quoted by Pertl, 1940)) were available

An importation of cholera into New York by way of an infected steamer arriving in October 1887 from Marseilles and Naples was averted by the rapid establishment of a correct diagnosis through laboratory methods. As maintained by Chambers, this had been the first occasion "to put bacteriology to practical use in combating an invasion by the scourge"

However although the disease failed to gain entry into North America, serious outbreaks during the period under review took place in South America (Argentina, 1886 and 1888 Chile 1887 and 1888)

Violent cholera outbreaks in 1892 in Afghanistan and Persia where the infection had found a temporary home led to an invasion of Russia via Baku. The infection once more reached Moscow and St. Petersburg and extended to the western confines of the country Continuing to exist in 1893 and 1894 (when serious outbreaks took place in the Volyniya Podolsk area) cholera is estimated to have claimed 800 000 victims in Russia during this period.

In 1892 cholera became widespread not only in Russia but also in Germany and France it assumed serious proportions only at Hamburg, however where an explosive outbreak, due no doubt to the distribution of unfiltered Elbe water by the waterworks took place The incidence of the disease in Hamburg and its suburbs where this water was utilized was therefore incomparably higher than that in two adjacent communities obtaining their water supplies from other sources, as is shown by the following data quoted by Sticker

Locality	*Number of inhabitants*	*Number of cases*	*Cases per mille*	*Number of deaths*	*Deaths per mille*
Hamburg and suburbs	579 904	19 891	34.3	7582	13.0
Altona	143 249	572	3.9	328	2.3
Wandsbeck	20 571	64	3 1	43	2.0

According to Hirsch, disastrous epidemics, connected probably with the serious exacerbation of the cholera situation in India in 1863 occurred in the "East Indies" (Indonesian archipelago) in 1863 and 1864, and in China and Japan in 1864-65

Prevalence of the infection in Thailand and Malaya led in 1873 to most serious inroads of cholera into Sumatra, Java, and Madura From Singapore, which seems to have acted as the main distributing centre the infection was also carried to Borneo and—directly or indirectly—to Manado on Celebes.

As far as the records collected by Wu Lien-teh go, the incidence of cholera in China was not particularly heavy during the last years of the pandemic Whether the disease was present at that time in Japan could not be established

However most serious outbreaks took place there in 1877 79 in which latter year 158 204 cases with 89 207 deaths were recorded

Fifth Pandemic (1881)

Although, notwithstanding the wide areas over which it held sway the fifth cholera pandemic, customarily stated to have lasted from 1881 to 1896 caused considerably less havoc than its predecessors, it marks a most important epoch in the history of this disease For in 1883-84 Koch studying the outbreaks then rampant in Egypt and Calcutta, was able to prove that, as had been suspected before by some advanced thinkers, cholera was the result of a specific gastro-intestinal infection

The main features of the pandemic may thus be outlined.

As the result of a serious exacerbation of the cholera situation in India, which led in 1881 to violent outbreaks in the Punjab especially in Lahore, the infection was carried to Mecca, where epidemics occurred in that as well as in the following year In 1883 cholera, possibly already imported during the previous year by pilgrims returning from Mecca (Hussein) became epidemic in Egypt, first at Damietta, situated at one of the mouths of the Nile not far from Port Said, where a fair was in progress at the time Spread initially by infected persons fleeing from Damietta, the disease broke out in Cairo Alexandria, and other places, claiming—according to Hussein—58 511 victims in the country

In Europe cholera remained during the early years of the pandemic practically confined to France Italy and Spain. In the first mentioned country it assumed epidemic proportions in April 1884 at Toulon, and this outbreak was soon followed by small epidemics in other places, including Marseilles and Paris, the total number of cases recorded during the year in France amounting to about 10 000 with a mortality of 50 % (Sticker) Cholera reappeared in France in 1885 mainly in localities afflicted during the previous year In 1887 a small outbreak (7 cases with 4 deaths) due to

Year	Countries affected
1888 1889	Indonesia (" Sunda Islands ")
1890	Indonesia
1891	Ceylon Thailand Straits Settlements, " Sunda Islands "
1896	Java

Cholera was also reported to be present in Thailand and Indonesia during 1897

In China the infection appears to have been particularly widespread from 1881 to 1883 as well as in 1888 and—to a lesser degree—in 1890 and 1895 while the presence of the disease in Korea in 1881 1888 1890 1891 and 1895 was noted by Wu Lien teh

Cholera epidemics in Japan during the period under review took place, according to Takano and co-authors, in 1881 (9000 cases) 1882 (more than 50 000 cases) 1885 (13 772 cases) 1886 (155 000 cases) 1890 (46 000 cases) 1891 (11 000 cases) and 1895 (over 55 000 cases)

An outbreak at Manila in 1882 was mentioned by Hirsch. The presence of the disease in the Philippines was also recorded in 1888 and 1889 (Kolle & Schürmann)

Sixth Pandemic (1899)

The appearance of the sixth cholera pandemic which may be said to have lasted until 1923 stood no doubt, in causal connexion with a most marked exacerbation of the cholera situation in India. It is true that, as pointed out by Sticker after the fifth pandemic the disease had not totally disappeared from western Asia and even Egypt but a local recrudescence from foci of the infection which possibly continued to persist in western Asia could at most, have been of auxiliary importance

This exacerbation of the cholera situation in India, commencing in 1899 led in 1900 to violent outbreaks in Calcutta and Bombay followed, until 1904 by a prevalence of the disease in the south of the sub-continent particularly in the Presidency (now State) of Madras as well as in the north That the infection possessed from the first a great tendency to spread beyond the confines of India is shown by a westward extension of cholera into Afghanistan and the Persian Gulf areas, taking place in 1900 and by the invasion of Burma and Singapore in 1901 which as is described on page 45 led to a further spectacular progress of the disease eastwards in the following year

Simultaneously with this spread to the east, cholera was carried in 1902 by the maritime route presumably by pilgrims who left Madras, to the port of Jidda and from there to Mecca where an outbreak beginning in the last week of February killed 4000 of the assembled multitude Though every possible precaution was taken it proved impossible to prevent

Cholera appeared in more than 250 other German communities besides Hamburg, but since the cases remained mostly sporadic, the total number in these places was restricted to 1048 with 607 deaths (Sticker) The reappearance of the disease in Germany during the following years also caused little havoc, the case incidence in 1893 being 915 (with 396 deaths) and that in 1894 when the eastern parts of the empire alone were involved amounting to 1004 (with 490 deaths)

As stated by in der Beeck, cholera appeared in 1892 in the northern departments of France (including Paris and its vicinity) but did not assume epidemic character In the following year it was mainly the southern parts of the country that were affected but in most of the 33 departments involved there were only sporadic cases or at most small outbreaks In 1894 sporadic attacks alone were noted in Toulon, Marseilles, and Paris

Though, as described by Chambers, eight badly infected ships arrived in New York harbour during 1892, adequate measures, facilitated by the opening of a city health laboratory rendered it possible to keep the infection at bay with the result that none of the 10 cases occurring in the city led to the establishment of a focus.

However as earlier in the pandemic, cholera appeared in South America, involving Brazil in 1893-95 Argentina in 1894 and 1895 and Uruguay in 1895 Still as stated by Sticker the infection invariably failed to entrench itself in these countries *(es blieb bei kraftlosen Anfängen die rasch von selber erloschen)*

In Africa, according to a table furnished by Kolle & Schürmann (1912) the following countries recorded cholera manifestations during the period under review

Year	Countries
1893	Tripolitania, Tunisia, Algeria, Morocco, French West Africa
1894	Sudan, Tripolitania, French West Africa
1895	Egypt, Morocco
1896	Egypt

However with the exception of the 1896 outbreak in Egypt, which caused over 16 000 deaths (Hussein, 1949) no considerable epidemics resulted.[1]

Throughout the pandemic, cholera not only continued to be prevalent in India, but appeared frequently or even perennially in the countries to the south-east or east of India. Besides outbreaks in Annam taking place, according to Wu Lien teh in 1882, cholera manifestations in South-East Asia were recorded by Kolle & Schürmann thus

[1] As stated by Sammons et al. (1944), in 1891 cholera occurred in the Setit River region of Eritrea. According to the same authors the last cholera outbreak in Ethiopia occurred in 1892-93.

caused no great havoc in Russia in 1911 and appears to have become sporadic during the following two years However, as shown by the adjoined table the disease again became widespread during the First World War, particularly in 1915 and having also been frequent in 1918 and 1920 showed a terrifyingly high incidence in 1921 1922 was still a bad cholera year but there was a marked decline in 1923 while only sporadic cases were noted in 1924 and 1925 Since then Europe has remained free from cholera

The orbit within which the prevalence of cholera during the period under review led directly or indirectly to the invasion of Western countries was far more limited than had been the case in previous pandemics The infection failing to penetrate into the Americas, the westernmost point reached by the disease was Madeira, which was affected in October 1910 *through the arrival of a steamer with unreported cases among immigrants* en route from Russia to South America Lasting until February, this epidemic claimed—according to the official records—600 victims among 1769 patients (Goldschmidt, 1910)

The visitations of western Europe by cholera during the sixth pandemic were restricted to the appearance of sporadic cases or in the rare instances where a spread of the infection did take place, to abortive outbreaks Thus, importation of the disease into Rotterdam in 1909 led to only 26 cases with 6 deaths among the population of the port, and to isolated occurrences in 18 other communities of the Netherlands (Sticker)

Though also causing considerably less havoc than on previous occasions, cholera at times during the sixth pandemic assumed quite serious proportions in central and south-eastern Europe In Italy where insignificant manifestations had been observed in 1909 in Apulia and at Naples, there were considerable outbreaks during the two years following. In the summer of 1910 the infection stated to have been recently imported via Brindisi *through gipsies coming from Russia*, claimed within a few weeks 1400 victims, but, as in 1909 remained restricted to the south of the country In the summer of 1911 cholera became manifest in all parts of Italy including Sicily, but assumed serious proportions in only a few of the numerous affected localities [1]

In Hungary where as in several other European countries cholera had been sporadic in 1909 a few epidemics took place in the following year and again in 1913 There as in Austria, importations of the infection through Russian (and later also through Serbian) war prisoners led to a quite serious cholera situation during the First World War (1914-16) In November 1914 Austrian troops who had come from the Volyniya Podolsk area, were instrumental in bringing the infection into Prussian Silesia, but no serious outbreak resulted However as in the case of Austria Hungary,

[1] The prevalence of cholera in Italy was presumably responsible for the appearance of an epidemic in Tunisia in 1911 in the course of which 733 cases occurred.

an invasion of Egypt where the disease imported in some manner never elucidated first became manifest in Asyut and then spread claiming within three months almost 34 000 victims (Hussein)

In what way the infection penetrated early in this pandemic into Russia is difficult to decide In the opinion of Sticker an invasion of Syria, taking place via the Sinai Peninsula in 1903 was responsible for the appearance of cholera in the same year not only in Palestine Asia Minor and on the Black Sea coast but also in Mesopotamia and Persia from which latter country the disease was imported in the spring of 1904 by caravans via Samarkand into Baku on the Caspian Sea. It is certain that cholera, becoming epidemic in this port in September 1904 spread in the same year still westwards into Transcaucasia, nothwards via Astrakhan up the Volga as far as Samara (now Kuibishev) and, according to Sticker also into western Siberia. In 1905 cholera remained restricted to the valleys of the Ural, Volga and Don rivers while the infection seems to have become quiescent in 1906 In the following year however the disease once more became epidemic in the Volga basin and spread in 1908 (*a*) as far as St. Petersburg and some of the Baltic ports (*b*) to several Black Sea ports, and (*c*) eastwards into Transcaspia, Turkestan, and Siberia. The cholera incidence slightly abated in 1909 but, as shown by Table II rose in 1910 to over 230 000 cases with almost 110 000 deaths, particularly severe epidemics being noted in Jekaterinoslav (18 894 cases), St. Petersburg (4591 cases) Kiev (4077 cases) and Orenburg (3355 cases) Cholera

TABLE II. CHOLERA INCIDENCE IN EUROPEAN RUSSIA FROM 1902 TO 1925

Year	Cases	Deaths	Year	Cases
1902	2 167	1 393	1914	9 715
1903 †			1915	66 455
1904	9 226	6 860	1916	1 600
1905	698	286	1917	130
1906	20		1918	41 586
1907	12 703	6 244	1919	5 119
1908	30 706	15 642	1920	29 615
1909	22 858	10 677	1921	207 389
1910	230 232	109 560	1922	86 178
1911	3 416	1 646	1923	114
1912	9	3	1924	Sporadic cases only
1913	324	149	1925	

After Otzecha ([illegible])
† No records available

In Persia cholera appears to have been rampant in 1906 but seems to have caused no great havoc when reimported from the north in 1908 (Sticker) Further manifestations of the disease in Persia were recorded in 1911, 1912, perennially from 1914 to 1919, and also in 1922 23

No doubt fomented by the First World War, cholera was rampant in Turkey in Asia in 1916 After the war outbreaks were recorded in Mesopotamia in 1918 and 1919 as well as in 1923 (Heggs, 1938) and in Palestine in 1918

As noted already the great activity displayed by the infection before the beginning of the sixth pandemic in India led to a rapid spread of the infection south-eastwards and eastwards The invasion of Burma and Malaya in 1901 was thus followed in 1902 by a spread of cholera over most parts of the Far East as far as China and Manchuria, Korea, Japan and the Philippines It is possible however that in some of the countries then invaded the new wave of infection merely reactivated already existing cholera foci. Be this as it may, it is certain that in most of the countries involved outbreaks continued to be frequent or even perennial though varying in extent and severity As far as can be gathered from the compilations of Kolle & Prigge (1928) Swaroop & Pollitzer (1952) and Wu Lien teh (1934) particularly serious outbreaks took place as shown in Table IV

As will be noted, in some of the countries concerned the cholera situation was particularly serious in 1908 1909 or in both years It is interesting to note that these bad cholera years were preceded by a period lasting from 1905 to 1908 during which cholera was particularly rampant in India, as shown by the following figures

Year	*Cholera deaths in India*	*Year*	*Cholera deaths in India*
1904	189 855	1907	400 024
1905	439 439	1908	579 814
1906	682 649	1909	227 842

The cholera mortality in India once more exceeded half a million annually in 1918 (556 533 deaths) and in 1919 (565 166 deaths) As shown by Table IV the cholera mortality in Java became quite unusually high during these two years while 1919 was a bad cholera year for Thailand and China. It is however difficult to decide whether these parellel developments indicate more than coincidences

Conclusion

When trying to deal in a summary manner with the geographical dis tribution of cholera throughout the world, it is far easier to refer to the few areas unaffected by this scourge than to enumerate the many countries where the presence of the disease has been recorded Generally speak ing, it may be maintained that the infection has not penetrated into the

transports carrying Russian prisoners of war were responsible for the importation of cholera into the interior of Germany where the disease became manifest in and near prison camps situated in various parts of the country Still, as stated by Krehnke, the number of cholera victims among the civilian population of Prussia from 1914 to 1918 totalled less than 60 Except among troops stationed in Turkey the incidence of the disease in the German army which had been systematically vaccinated against cholera, remained low

As shown by Table III cholera outbreaks during the period under review were quite frequent and often serious in the Balkan peninsula where the spread of the infection was facilitated by the local wars taking place in 1912 and 1913 and also to some extent by the First World War

TABLE III CHOLERA OUTBREAKS IN THE BALKAN PENINSULA, 1910-22

Year	Countries affected
1910	Greece Turkey
1911	Bulgaria, Greece Montenegro, Romania Serbia, Turkey †
1912	Bulgaria, Turkey †
1913	Bulgaria, Greece Romania,† Serbia,† Turkey
1914	Bulgaria, Serbia †
1915	Serbia
1916	Albania, Bosnia and Herzegovina † Corfu Turkey
1917	Turkey (Istanbul)
1918	Macedonia
1919-20	Turkey (Istanbul)
1922	Greece (Athens) Romania

Largely based on data from Kolle & Schürmann (1912) and Kolle & Prigge (1928) Greece is included for 1913 on the authority of Savas (1914).

† Major outbreak

In south west Asia during the period under review cholera manifestations continued to be frequent in Arabia and Persia A particularly violent outbreak, due apparently to the arrival of pilgrims by ship via Odessa arose in Mecca at the end of 1907 and claimed in 1908 more than 25 000 victims in the Hejaz (Sticker) Further appearances of the disease in Arabia were recorded in 1909 (Hejaz) 1910 (Mecca) 1911 (major outbreak involving Mecca) and 1912. According to Duguet, Mecca and the Hejaz as a whole have remained free from epidemic cholera since then

northernmost and southernmost parts of the globe Accordingly it may be noted that in Asia northern Siberia and Kamchatka have been spared and the same holds true of the most northern parts of western Europe (Iceland the Faeroe, Shetland, and Orkney Islands the Hebrides, Norway north of Bergen and Lapland) as well as the North American regions beyond the 50th parallel including Newfoundland (a major part of which, however lies south of that degree of latitude) and Greenland Similarly cholera, though occasionally imported into South African ports, for exam ple in 1890 into Durban (Clemow), invariably failed to entrench itself, while the countries on the west coast of Africa south of Portuguese Guinea appear to have remained altogether free from the infection In South America also cholera has remained absent from the southernmost parts of Chile and Argentina and from the Falkland Islands However, the appearance of the disease in the Archangel government situated on the White Sea in European Russia forms an interesting exception to this rule

Besides the areas mentioned above some islands such as St Helena and Ascension and the Bermudas situated well away from continents, have remained exempt from cholera invasions

It is no doubt true that cholera was far more frequent in areas situated north of the equator than in the southern hemisphere but as shown by the frequency of violent manifestations of the infection in Indonesia and the repeated appearance of the disease south of the line in Africa and America, this unequal distribution cannot be due to factors of a strictly epidemiological nature

An interesting question arising in this connexion is whether cholera ever gained an entry into the Pacific areas As noted above, the claim of an inroad of the infection into western Australia deserves little if any, credence Lack of other pertinent information makes it also difficult to accept the statement of Simmons et al (1944) that the disease was present during the nineteenth century in the Japanese Mandated Islands (Marianas or Ladrone Islands, the Caroline Islands and the Marshall Islands) while the true nature of a few cases reported there in 1929 30 seems rather questionable However it deserves attention that as asserted by Sticker, cholera was imported in 1893 into (German) New Guinea and continued to occur there without causing major havoc and that in 1896 the infection also gained a foothold in the Bismark Archipelago and the island of New Britain areas situated comparatively near the frequently cholera affected Indonesian archipelago

Although fairly reliable figures are occasionally available, it is—as justly maintained by Haeser—altogether impossible to determine with even approximate accuracy the global mortality caused by cholera during the above-described pandemics Nor is it possible to arrive indirectly at a reliable estimate by establishing in a generally valid manner the relation existing between the incidence of the disease or the fatalities caused by

TABLE IV YEARS OF HIGH CHOLERA INCIDENCE IN SOUTH-EAST ASIA†

Year	Burma	Indo-China	Thailand	Federation of Malaya	Java	China	Korea	Japan	Philippine Islands
1902								8 164	
1903	8 233								
1904									
1905	5 347								
1906	7 872								6 067
1907	7 678							1 702	
1908	11 911								17 770
1909	11 389								8 568
1910								1 957	7 202
1911		3 833							
1912	7 186	12 028			5 511			1 663	
1913					2 040				
1914									
1915	17 697	6 326							
1916		6 987						6 260	7 966
1917									8 723
1918					9 664				6 340
1919	13 260	4 798	10 277		8 861				17 637
1920			2 748					3 428	
1921		2 838							
1922	5 047								

† Where mortality records are available, figures are given.

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it, and the number of the inhabitants of the affected localities. This is impracticable not only because the percentage rate of cases and deaths was apt to show marked differences in different outbreaks but also because quite often a panic flight of the people from cholera stricken places led to a great reduction of the individuals actually at risk, while in other instances the presence of pilgrims or other non-residents resulted in a marked increase of the fuel available for the infection

However even though exact information is often lacking there can be no doubt that, as asserted by Haeser the loss in lives caused by cholera during the rather short course of its known history must be counted in millions Great as this death toll must have been it cannot compare in any way with the mortality caused in the past by plague which is supposed to have killed 100 million people during the pandemic taking place in the sixth century and to have caused the death of 25 million in Europe alone at the time of the Black Death It is, however of great importance to note that, as indicated by the figures for India given in Table V there is reason to assume that the number of fatalities caused by cholera is now greatly in excess of the death toll exacted by plague

TABLE V DECENNIAL MORTALITY FROM CHOLERA AND PLAGUE IN INDIA, 1909-48

Decade	Cholera deaths	Plague deaths
1909-18	347 068	422 153
1919-28	250 246	170 972
1929-38	168 190	42 288
1939-48	202 195	21 797

After Swaroop & Pollitzer (1952) and Pollitzer (1954)

It must be admitted that the great reduction in the incidence of plague —evident not only in India but also in most other still-affected parts of the world—which set in long before it was possible to implement the improved methods for treatment and control now available is due largely to intrinsic causes There can be no doubt, however that increasing use of these procedures is now bound to speed up the reduction of the disease. In the case of cholera which, in India at least, has so far shown no signs of a really satisfactory decrease methods of treatment and control combining easy application with full efficiency must still be sought. Hence while in most respects the plague problem may be considered a *res gesta*, the many still unsolved problems of cholera continue to call for urgent attention

Chapter 2

WORLD INCIDENCE *

GENERAL OBSERVATIONS

In the preceding chapter the history of cholera in the world has been traced from ancient times up to the year 1923 This period covers two main phases in the history of the disease, namely that prior to 1817 during which cholera was confined to the East, if not almost exclusively to India, and a second period lasting from 1817 to about 1923 during which pandemics originating from India spread to countries lying east and west of India and in some cases swept over several continents of the world. During the following period, lasting from 1923 up to date cholera became once more almost entirely a disease of the East, because during that time no major spread westwards beyond Afghanistan took place, with the exception of an invasion of Iran in 1939 and isolated epidemics which appeared in Egypt and in Syria in 1947-48 believed to have been due to extraordinary conditions created by the Second World War

The appearance of the 1817 cholera pandemic at a time when British troops were occupying some important areas of India and were consequently severely affected by the disease, as well as the occurrence of a series of further pandemics causing world wide havoc have stimulated public health workers to record, in a continuous and gradually improved manner the information bearing on the epidemiology of the disease. Historical facts relating to the incidence of cholera from year to year since 1817 have therefore been studied with considerable care and thus an increasing volume of valuable information which now covers a period of almost seven score years, has become available. Based on these data a number of historical accounts, referred to in the first chapter have been published as well as statistical studies on the prevalence and mode of spread of the disease such as those of Rogers (1928) and Russell & Sundararajan (1928) Such studies based as they were on an accumulating fund of statistical

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The appearance of the 1817 cholera pandemic at a time when British troops were occupying some important areas of India and were consequently severely affected by the disease as well as the occurrence of a series of further pandemics causing world wide havoc, have stimulated public health workers to record, in a continuous and gradually improved manner the information bearing on the epidemiology of the disease. Historical facts relating to the incidence of cholera from year to year since 1817 have therefore been studied with considerable care and thus an increasing volume of valuable information which now covers a period of almost seven score years, has become available Based on these data a number of historical accounts referred to in the first chapter have been published as well as statistical studies on the prevalence and mode of spread of the disease, such as those of Rogers (1928) and Russell & Sundararajan (1928) Such studies based as they were on an accumulating fund of statistical

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knowledge have brought out certain well-established epidemiological patterns of the disease concerning its seasonal, geographical, and climatological variations, and its general mode of spread In this chapter we propose first to comment briefly on some of these well-established facts concerning cholera incidence so that their knowledge may lead to a better understanding of the occurrence and spread of cholera in the world since 1923

Existence of Cholera Endemic Foci

As has been pointed out in the first chapter each major epidemic spread of cholera since 1817 appears to have originated in the endemic home of the disease in India, where the infection has been entrenched since long before 1817 probably even since immemorial times.

Recent studies of the cholera mortality statistics, undertaken with the aim of demarcating the areas which truly harbour endemic foci of cholera in India, have drawn a distinction between

(1) Regions of large size where epidemics occur only occasionally i.e. which remain free from infection for considerable periods, as for example the present States of the Punjab Delhi, Madhya Pradesh, Hyderabad, Mysore, and Bombay

(2) Areas where the disease continues to be present at a fairly high level from year to year and where it may at times assume epidemic proportions —examples are the State of Bengal, the coastal areas of Orissa, and certain districts of Bihar and Assam.

The areas of the second category are generally considered to be endemic to a varying degree, even though in some of the highly endemic areas of this kind it may not be possible with the available laboratory techniques and facilities, to trace the spread of infection from patient to patient either directly or indirectly particularly during the interepidemic periods.

On the basis of cholera mortality statistics relating to the period 1901-45 Swaroop & Pollitzer (1952) described the geographical distribution of the areas harbouring the endemic foci (see Fig. 1) The largest of these foci has its centre in Bengal (in both East and West Bengal now forming part of Pakistan and India respectively) in the deltaic region of the Ganges and the Brahmaputra, extending eastwards into Assam, westwards into Bihar and possibly the eastern districts of Uttar Pradesh Other endemic foci of lesser magnitude are found in the deltas formed by the Mahanadi in Orissa State, the Cauvery the Kistna and the Godavari in Madras State, and possibly also in the Irrawady delta in Burma. Common factors to all these foci are that they are situated in close relation to surface-water systems that they are densely populated areas at or near the coast, and that they lie at an altitude hardly exceeding 50 feet above sea level. There is reason to assume that, possibly owing to improvements in sanitation,

FIG 1 CHOLERA ENDEMICITY LEVEL IN INDIA AND PAKISTAN 1901-45



The endemicity level is shown in relation to the river system by the variation in the density of the dots This level is expressed by the average annual cholera death-rate during the fifteen years of lowest incidence in the period 1901-45.

some of these foci have shrunk in size within recent years, so that presumably the endemic zones from which the infection could start its wide sway in the past were indeed extensive.

Because some of these foci particularly those in and near Bengal, have been *definitely known to exist since 1817* and also because in no other part of the world has cholera succeeded in establishing itself in such a permanent manner the conclusion that the major endemic focus in Bengal has all along constituted the reservoir of the infection and the starting point of the cholera pandemics, has often been reached, e.g., by Bryden (1874) and by Macnamara (1876) While the demarcation of the endemic foci in India supports this idea in general any attempt to trace the origin of individual outbreaks to any single locality within this vast area would be futile In fact, one must presume that epidemic outbursts in these regions lead to the production of a great volume of infection in different localities, sometimes even simultaneously at places distant one from another and that under favourable climatic conditions the disease then spreads in a wave like form to areas generally free from cholera.

Influence of Pilgrimage Centres and Festivals in the Spread of the Disease

India is a land of pilgrimages and shrines. Religious assemblies attracting thousands of devotees from various parts of the country take place at frequent intervals In the past, the sanitary conditions at such congregations were appalling and, to make matters worse, almost invariably the ritual obliged the pilgrims to bathe in a common place in a river or tank Under these circumstances, it is not surprising to find that such congregations often resulted in violent outbursts of cholera in non-endemic areas, the limit of spread of these epidemics depending to some extent upon the number and range of movement of the pilgrims, and upon favourable climatic factors Characterizing the danger created by the pilgrimages, Rogers & Megaw (1952) stated

> Each year about 20 000,000 pilgrims make long journeys in India to visit sacred, but often very insanitary shrines and they frequently disseminate the disease over large areas.

It is undeniable that these festivals, specially if held during seasons favourable to the propagation of the disease, play a most important role in the *spread* of cholera epidemics. At the same time however it has to be pointed out that the influence these gatherings exert in *maintaining* endemic foci in India is perhaps relatively unimportant. Swaroop & Raman (1951) studying the geographical location of such centres throughout the country and the number of pilgrims they attract, reached the conclusion that " an explanation for endemicity of the disease must be sought elsewhere than in the occurrence of fairs and festivals "

Seasonal Factors Influencing the Spread of Cholera

Ever since statistical data on the incidence of cholera have been collected it has been found that the disease shows a consistently similar and well-marked seasonal variation in individual parts of India, together with striking variations from area to area. It is of particular importance that almost each year the disease has shown in Bengal a tendency to become frequent during the period of September to November and to reach a peak in December and January followed by another seasonal increase in March-April (see Fig. 2) There is however considerable variation in the individual regions of Bengal.

Another important feature is that, as one proceeds westwards from Bengal, there is a marked tendency for the cholera peak to appear later so that the winter months prove to be unfavourable to the spread of the infection in the contiguous States of Bihar and Uttar Pradesh (formerly

the United Provinces) Thus, as shown by Fig 2, the seasonal cholera incidence reaches in these two States its peak in spring while in the Punjab the disease shows a relatively high incidence during the monsoon months of July and August

FIG 2. MEAN MONTHLY CHOLERA DEATHS FOR FIVE INDIAN PROVINCES (1925-48)

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in the pandemic commencing in 1817 when cholera, after a temporary quiescence during winter in Bengal and Bihar, became epidemic in the United Provinces (Uttar Pradesh) some time during the month of March 1818 and then spread from there in various directions. From 1923 onwards, the seasonal spread of cholera has followed the same " time table " unless the occurrence of some fair or festival accelerated the progress of the disease.

The history of the spread of cholera from Bengal is usually therefore, characterized by the rise of a seasonal wave in the endemic home, followed by a recrudescence in Uttar Pradesh, from where the disease is carried northwards through the Punjab along the land route to Afghanistan, Iran etc. Each year the incidence of cholera in the Punjab subsides during the winter months, but it is of interest to note that during these months a spread of the infection into Kashmir was apt to take place as exemplified by the description of the 1925 26 epidemic in that State.

The spread of cholera from Bengal through the United Provinces and the Punjab north westwards through Afghanistan has followed what Bryden (1874) called the " northern epidemic highway of the disease " This author also described a " southern epidemic highway " along which cholera spread from the United Provinces through central India south wards to the States of Madras and Bombay sometimes reaching Ceylon and being carried by sea from Bombay to the Western world Ever since records have been available the disease in its westward spread from India has continued to follow these general routes

A surprising feature of the spread from Bengal is that cholera has never reached southern India by what is the shortest and direct route geographically through Orissa along the east coast, the more so as an important endemic focus of the disease is located at the delta of the Mahanadi River The explanation offered in 1871 by Cornish (quoted by Rogers, 1928) was " that the disease was checked by the sparsely inhabited hill tracts reaching down close to the coast at this point "

The history of the spread of cholera from India to countries eastwards is a record of maritime transmission seaports in that part of the world being liable to primary outbreaks followed by progress of the infection to the hinterland Small coastal craft have undoubtedly played a part in the local diffusion of the infection

Disappearance of Cholera from the West

The figures of Table VI showing the mortality due to cholera throughout the world from 1900 to 1954 well bear out the statement made at the beginning of this review that, apart from some occasional inroads taking place as a rule under extraordinary circumstances, from 1923 onwards the manifestations of the disease remained virtually restricted to India and some countries to the east of it.

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It is curious to note that besides marking the end of the last cholera pandemic, the year 1923 is also remarkable in so far as the cholera mortality then reported in India, though still considerable, was the lowest on record for any year since the beginning of the century In fact, during two months of 1923 not a single cholera death was reported from an area comprising about half of India including Uttar Pradesh and States situated on its west and south-west Seeing, however that (*a*) cholera again became far more rampant in India in some of the subsequent years without spreading westwards and (*b*) it had disappeared from most Western countries well before 1923 not much significance can be ascribed to the low incidence of the disease in India during that year The true rather complex causes of the disappearance of cholera from the west will be discussed later

RECENT INCIDENCE OF CHOLERA[1]

Cholera in Russia

The figures in Table VII showing the case incidence of cholera in Russia from 1823 to 1926 though incomplete as far as the period prior to 1904 is concerned serve to indicate the relative magnitude of the manifestations of the disease during the various invasions of the country It will be gathered that, following the peak year of 1921 during which the situation became almost as serious as in 1910 cholera was still quite frequent in 1922, but that then a rapid decline set in

Of the 114 cases reported during 1923 73 occurred during an outbreak in the city of Rostov-on the Don the remainder being sporadic occurrences in widely separated localities A detailed account of the history of cholera in Rostov-on the Don from 1920 to 1925 was published by Barikine & Cazeneuve (1925)

After the disappearance of cholera from Russia in 1926 no outbreak of the disease has occurred in any European country with the possible exception of a small and rather doubtful epidemic during the Second World War in the German Army in the Ukraine. According to Stowman (1945)

" A statement was made by Dr Heilmeyer of Jena at the meeting of the Deutsche Gesellschaft für Innere Medizin at Vienna 10-14 October 1943 to the effect that cholera had been no problem to the Germans during the present war because the only outbreak which had occurred had been one of 78 severe cases in the German Army in the Ukraine [no date given]. The word cholera was not qualified, and it is barely possible that he meant cholera nostras, which, however is a rather vague term, usually covering outbreaks of food poisoning due to Salmonella or Proteus infection.

[1] This review is based mainly on a study of the final figures of cholera incidence available up to the end of the year 1954 However in order to illustrate the most recent trend of the infection, a map has been included showing cholera distribution in the world during the period 1800-1957 and the incidence of cholera in 1957 (Fig. 3).

IN VARIOUS COUNTRIES FROM 1900 TO 1954

Taiwan (Formosa)	Japan	Philippines	USSR	Egypt	Iraq	Iran	Syria	Austria	Belgium	Germany	Hungary	Italy	Spain	Years
—			—	—				—		—		—	17	1900
1			—	—				—		—	—	—	17	1
613	8 164	80 302	1 303	34,595				—		—	—	—	—	2
1	140	29 294	—	3				—	9	—	—	—	2	3
1	51	84	6 850	—				—	6	—	—	—	9	4
—	34	1 417	786	—				—	6	86	—	—	1	5
—	29	6 067		—				—	33	—	—	—	—	6
	1 702	718	6 424	—				2	2	—	—	—	2	7
—	297	17 770	15 542	—				8	—	1	—	—	—	8
—	158	8 566	10 677	—				—	10	9	—	—	—	9
8	1 957	7 702	109 660	—				6	10	11	302	804	—	10
—	4	166	1 646	[illegible]				39	2	—	252	6 145	44	1911
256	1 683	—	3	—				—	—	—	1	41	—	12
—	22	217	149	—				22	—	—	722	—	—	13
—	4	2 395	761	—				114		43	1 153	—	—	14
—	—	820	859	—				85		1 804	4 057	235	—	15
18	6 260	7 966		1				8		61	16	11	—	16
1	641	7 691		2				38		38	4	—	—	17
1	—	4 702	12 927	15				1		63	2	—	—	18
2 696	916	18 367	E	—				4	—	2	2	—	—	19
1 675	3 426	1 387	E	—	—			4	—	—	1	—	—	20
—	39	54	E	—	—			5	—	2	—	—	3	1921
—	542	[illegible]	E	—	—	2		—	—	—	—	—	—	22
—	31	11		—	1 110	1 029	—	—	—	—	1	—	—	23
—	—	26		—	—	2	—	—	—	—	1	—	—	24
3	363	588		—	—	—	—	—	—	—	—	—	—	25
16	13	238		—	—	—	—	—	—	—	—	—	—	26
—	3	3	—	—	1 063	593	—	—	—	—	—	—	—	27
—	1	8	—	—	—	—	—	—	—	—	—	—	—	28
—	114	—	—	—	—	—	—	—	—	—	—	—	—	29
—	2	3 079	—	—	—	—	—	—	—	—	—	—	—	30
1	—	784	—	—	1 548	165	—	—	—	—	—	—	—	1931
16	1	447	—	—	—	—	—	—	—	—	—	—	—	32
—	—	1 807	—	—	—	—	—	—	—	—	—	—	—	33
—	—	636	—	—	—	—	—	—	—	—	—	—	—	34
—	—	5	—	—	—	—	—	—	—	—	—	—	—	35
—	—	1	—	—	—	—	—	—	—	—	—	—	—	36
—	11	1	—	—	—	—	—	—	—	—	—	—	—	37
—	10	—	—	—	—	—	—	—	—	—	—	—	—	38
—	1	—	—	—	—	307	—	—	—	—	—	—	—	39
—	5	—	—	—	—	—	—	—	—	—	—	—	—	40
—	—		—	—	—	—	—	—	—	—	—	—	—	1941
—	—		—	—	—	—	—	—	—	—	—	—	—	42
E	1		—	—	—	—	—	—	—	—	—	—	—	43
	—		—	—	—	—	—	—	—	—	—	—	—	44
	—		—	—	—	—	—	—	—	—	—	—	—	45
2 210	528	—	—	—	—	—	—	—	—	—	—	—	—	46
—	—	—	—	20 462	—	—	—	—	—	—	—	—	—	47
—	—	—	—	10	—	—	18	—	—	—	—	—	—	48
—	—	—	—	—	—	—	—	—	—	—	—	—	—	49
—	—	—	—	—	—	—	—	—	—	—	—	—	—	50
—	—	—	—	—	—	—	—	—	—	—	—	—	—	1951
—	—	—	—	—	—	—	—	—	—	—	—	—	—	52
—	—	—	—	—	—	—	—	—	—	—	—	—	—	53
—	—	—	—	—	—	—	—	—	—	—	—	—	—	54

= Figures not available

— = No cholera death recorded

TABLE VI CHOLERA DEATHS RECORDED

Years	India & Pakistan [a]	Burma	Ceylon	Thailand	Indo-China	Java	China	Shanghai	Hong Kong	Korea	Malaya	Singapore
1900	806 698	3 481			E							194
1	267 657	3 552	95		E	7 637			9			120
2	222 235	1 901	116		E	4 790	E	1 149	433			737
3	304 621	8 233	28		E	E	C	165	6			184
4	189 655	2 980			E			1				3
5	439 439	6 347			C			—	5			16
6	682 649	7 672	E		C			197	1			171
7	400 424	7 678					E	699	30			167
8	579 814	11 911	30		C		E	9	39			127
9	227 842	11 389			C	C	E	4	2			77
10	428 440	2 011					E	—	7			130
1911	349 614	4 191	277	E	3 833			—	3			235
12	400 563	7 166		E	12 028	5 511	E	1 321	1			114
13	290 476	4 339		E	281	2 040	C	—	40			79
14	278 657	2 073			489	1 144	C	350	16			211
15	386 875	17 697			6 326	1 118	C	—	11			6
16	286 374	1 673			6 987	1 108	E	100	10			8
17	265 088	1 914		78	1 439	327		—	—			7
18	556 633	4 269		42	1 456	9 864		—	—	—	233	—
19	566 166	13 260	419	10 277	4 798	8 861	E	680	42	11 084	237	68
20	126 744	3 396	—	2 748	1 035	17	E	144	6	13 568	14	32
1921	448 617	3 791	—	40	2 838	1	C	122	5	1	—	1
22	116 632	5 047	—	80	992	28	142	101	—	23	—	1
23	71 514	1 488	—	108	143	—	67	94	1	—	C	—
24	285 624	6 083	14	61	73	—	2	—	—	—	C	4
25	113 713	1 932	89	2 076	96	—	E	99	2	—	C	—
26	131 969	6 182	64	7 133	14 425	—	E	466	1	159	C	1
27	300 182	4 628	3	1 138	24 201	10		124	3	—	74	14
28	344 096	7 209	3	1 379	4 905	1		9	—	—	—	6
29	287 464	7 970	19	2 084	3 955	—	E	135	—	15	—	—
30	336 661	661	—	64	2 406	—		6	—	—	—	—
1931	220 375	534	6	16	1 582	—		27	—	—	—	—
32	66 137	1 082	—	13	363	—	E	205	156	33	—	—
33	68 139	179	—	22	190	—		—	—	—	—	—
34	196 866	622	1	6	94	—		2	—	—	—	—
35	210 304	6 858	22	917	86	—		—	—	—	—	—
36	158 736	985	44	3 134	61	—		—	—	—	—	—
37	99 054	3 492	2	5 967	8 892	—		696	1 082	1	—	—
38	236 143	568	—	3	6 626	—		2 106	363	32	—	—
39	97 665	1 468	5	9	1	—		57	448			
40	86 133	6 916	1	148	314	—	43 135	46	626			
1941	228 141		3	—	39	—	71	49				
42	218 496		42	—	14	—	9 521	136				
43	459 930		47	1 410		—	6 318	—				
44	294 625		9	1 078		—	350	—				
45	278 498	6 220	5	3 954		—	5 201	143				
46	122 817 [b]	2 482	31	4 660		—	15 460	353	246		182	
47	131 758 [b]	500	—	2 036	2 108	—	261		—		—	—
48	194 920 [b]	33	—	15	1 577	—	3		—		—	—
49	95 410 [b]	135	2	1	20	—	—		—		—	—
50	120 627	2 448	—	—	6	—	—		—		—	—
1951	58 844	4 771	—	—	19	—			—		—	—
52	74 748	236	—	—	17	—			—		—	—
53	107 758 [c]	9	11	—	7	—			—		—	—
54	16 133	19	1	—	4	—			—		—	—

a Former British provinces
b Excluding figures for East Bengal
c Provisional figures

E = Cholera prevalent in epidemic form.
C = Local outbreaks or sporadic cases.

The epidemic in Iraq causing 1503 cases and 1110 deaths, began in August 1923 reached its peak in September (773 cases, 585 deaths), and disappeared completely from the country by the end of the same year The most seriously affected city was Basra with 585 cases and 438 deaths After the 1923 epidemic, two more outbreaks occurred in Iraq one in 1927 (1063 deaths) and the other in 1931 which also began in Basra. The cause of the outbreak commencing in July 1931 was the disembarkment of two cholera patients coming from the infected port of Bombay An epidemic immediately developed at Basra and lasted until the middle of November The infection also spread along the Tigris and the Euphrates the total mortality in Iraq amounting to 1548 Since 1932, that country has remained free from cholera.

Iran

In Iran, the infection was present in a mild form during 1922, when 28 cases and two deaths were recorded The epidemic outbreak of 1923, causing 1029 deaths, was mostly confined to the ports of Abadan (992 deaths) and Mohammerah (27 deaths) and in its spread was a continuation of the epidemic that had had its origin in Basra, Iraq An extension took place along the Karun River as far north as Khurramabad. Three more epidemic outbreaks have occurred in Iran during the period under review namely during 1927 (593 deaths) 1931 (165 deaths) and 1938-39 (307 deaths) During 1938 the incidence of cholera was relatively high in the Punjab India, from where the disease spread through Afghanistan to Iran The 1939 outbreak in Iran is known to have lasted only two months and came to an end early in August. From 1940 onwards Iran has remained free from cholera.

Afghanistan

Cholera appeared in north India in several districts near the Afghan border in May 1930 and continued till October of the same year In Afghanistan, an epidemic broke out in July 1930 in the valley of the Kabul River and affected the major towns of Kabul, Jellalabad and Charikar Further south the disease spread in July to Ghazni (where over 160 cases were reported in two days) reaching Kandahar and Makur Kalat in August. In the latter towns it is known to have prevailed with "marked severity" and to have abated in September 1930 The total number of cases and deaths is not known.

After six years another outbreak of cholera was reported in Afghanistan during 1936 According to the 1938 report of the Public Health Commissioner with the Government of India the disease was also present in 1937 Becoming once more epidemic, cholera claimed 2141 victims in 1938 while during the following year 849 deaths from this disease were recorded in the country As stated by Biraud & Kaul (1947) "very small outbreaks" of cholera took place in Afghanistan in 1941 and in 1946 (35 cases)

TABLE VII CHOLERA IN EUROPEAN RUSSIA, 1823-1926

Year	Cases	Year	Cases	Year	Cases
1823	392	1859	4 931	1907	12 703
1829	3 590	1860	Isolated cases	1908	30 705
1830	68 091	1861	Isolated cases	1909	22 858
1831	466 457	1865	13 397	1910	230 232
1832	1 177	1866	208 853	1911	3 418
1833	14 428	1867	6 [illegible]45	1912	9
1834	Isolated cases	1868	310	1913	324
1837	Isolated cases	1869	1 278	1914	9 715
1838	Isolated cases	1870	21 664	1915	66 455
1847	180 848	1871	322 711	1916	1 600
1848	1 742 439	1872	310 607	1917	130
1849	15 223	1873	9 943	1918	41 686
1850	54	1892	620 051	1919	5 119
1852	10 428	1893	106 600	1920	29 615
1853	249 788	1894	65 140	1921	207 389
1854	28 052	1895	30 811	1922	66 178
1855	331 025	1896	46	1923	114
1856	11 687	1902	2 167	1924	9
1857	1 811	1904	9 226	1925	11
1858	3 649	1905	698	1926	1
		1906	20		

" The clinical description, however seems to tally with that of Asiatic cholera, and so does the treatment—intravenous injections of saline solutions. The fatal cases, the proportion of which is not given, but appears to have been considerable, showed pronounced uraemia. It was proved that the disease was not waterborne. Bacteriological and serological examinations showed atypical milder cases, some of them ambulatory among the contacts. This may occur also in epidemics of Asiatic cholera.

" It will be recalled that following the epidemic of Asiatic cholera in the Ukraine 1918-1922, choleriform vibrios and phosphorescent vibrios were still found in 1925 in the waters of the Don at Rostov and in fish and shrimps taken in these waters. It was found that ingestion of these vibrios caused an acute cholera attack and that typical cholera vibrios appeared in the stools. The case mortality rate of such infections was lower than in classical cholera. Abortive cases and carriers were found. At any rate, the infection can hardly be called true Asiatic cholera"

Cholera in Non-European Countries West of India

Iraq

Among Asiatic countries lying west of India only Iraq and Iran recorded high figures during 1923

"The source of the infection still remains a mystery for practically at the same time, cholera was discovered over a wide area between Cairo and Abou Sueir a distance of 132 kilometres. Although cholera was prevalent at the time in Pakistan and many British service-men were transferred from India to Egypt by air cholera did not break out in the Canal Zone, but in areas where no British were stationed."

Lasting for a period of about three months the epidemic led to 32 978 cases, with 20 472 deaths. Exhaustive accounts of the manner of its spread, its epidemiology, and the control measures adopted have been given by Shousha (1948) Khalil (1948) and by Biraud & Kaul (1947)

It is of interest to note that while, in the past, Egypt used to serve as a stepping stone for the spread of cholera from Asia to Europe, the 1947 epidemic did not lead to a westward spread of the infection either by ship or by aircraft On the other hand while there is no statistical evidence to suggest that cholera has been or is endemic in Egypt, the 1947 outbreak proved that, once the infection has been imported, its spread is facilitated by the environmental conditions prevailing in the rural areas of the country

As stated by Stowman (1945)

"The powerful sanitary barrier set up in the Red Sea and at the Suez Canal has functioned to full satisfaction as far as cholera is concerned. Not a single cholera case has come through to Europe that way for 30 years."

Syria

A cholera outbreak of unknown origin commenced in Syria on 20 December 1947 i.e after the disease had almost disappeared from Egypt Seven confirmed cases were first reported from two neighbouring villages in the Hauran Province, the infection later spreading to three more villages in the same province All five villages were on the main Dera Damascus road. Altogether 45 cases and 18 deaths were reported (*Lancet* 1948) Three non fatal cholera cases occurred in Syria in 1948

Arabia

Prior to the period 1923-53 cholera used to be the scourge of pilgrims journeying to the Hejaz. Duguet (1931) stated that there had been 27 epidemics during the Mecca pilgrimage in the previous 81 years and that the Hejaz was indeed a relay station of cholera in its progress from the east towards the west. At times, the cholera mortality in Mecca assumed grievous proportions, the death roll at the peak of the 1902 outbreak, for instance amounting to 800 to 1000 daily During the 1907 08 pilgrimage more than 20 000 cholera deaths occurred.

The last epidemic in Mecca took place during the years 1910-12. Since 1913 the pilgrimages have remained free from cholera, with the possible exception of one which took place in 1930 and which was considered "infected" although no case or death from cholera was reported in the Hejaz because in May a pilgrim returning from Mecca was landed at Massawa Eritrea, with symptoms of the disease

Egypt

In the post war years the Western countries remained free from cholera until 1947 when a minor outbreak lasting until 1948 commenced in Syria, and a severe epidemic spread in Egypt

Cholera had been absent from Egypt since 1919 when it made a sudden appearance on 22 September 1947 While previous outbreaks in that country had usually been due to an importation of the infection through pilgrims returning from Mecca, this did not hold true of the 1947 epidemic, which broke out before the pilgrims had returned, and nearly a month before the great festival of 20 October

The outbreak began at El Korein, an important trading centre of some 15 000 inhabitants on the eastern fringe of the Nile delta, situated close to the canal which provides drinking water for the cities and villages along the Suez Canal at a time when merchants had congregated from all provinces to attend the annual date fair

In addition, some 6000 workmen engaged in construction work near by were at the time billeted in El Korein village, and it is believed that the panicky flight of this floating population immediately after the appearance of the disease and before local quarantine could be enforced accounted for the rapid diffusion of the infection In three days the presence of cholera was noted at Cairo on 27 September it had reached Ismailia, and by the 29th the whole of the Kalyubiya and Dakahliya Provinces as well as Shar kiya were affected, besides the Suez area. In three weeks all provinces of Lower Egypt were involved, and by October 1947 the provinces of Upper Egypt had been reached by the infection However by December cholera had practically disappeared from Egypt.

While it was impossible to ascertain how the outbreak originated, the Egyptian health authorities were of the opinion that the infection had in all probability been imported from India. In this connexion the following extract from the *Lancet* (1947) may be quoted

"The source of the epidemic is still obscure and no official statement concerning its discovery has been made in Egypt. But many first-hand observers think they can trace the origin of the infection to Egyptian labourers infected by aeroplanes coming from India to British Army aerodromes, where these labourers work. It seems that some of the first cases were actually engaged in these airfields, which—until 6 October—were not under the control of the Egyptian Quarantine Department. There is also a coincidence between the Egyptian epidemic and that which started on 15 August 1947 in the Punjab after its partition between Pakistan and Hindustan, and the migration of about 5 000 000 persons which followed it. In support of this view they point to the return from India of British troops, who used the Suez Canal region as a quarantine station and remained there with their Indian retinue for 2 weeks before departing for England. British authorities, however, deny that there is any relation between the present epidemic and their Forces. It has certainly been established that the epidemic is in no way related to pilgrimage."

An editorial in the *British Medical Journal* (1951) contained the following statement

kampong containing [illegible] inhabitants. All the cases had symptoms which clinically resembled cholera and the first cases died within 12 to 24 hours of their onset. The mortality rate appears to reach [illegible] %, but there may be a number of unrecognized mild cases.

"A point of some interest is the finding of carriers in a percentage of 10-15% among contacts of cases and a percentage of 0.5% in infected kampongs. Of special importance was the finding in one kampong, which as far as could be ascertained, was quite free from infection, of 15 carriers among 1,000 persons examined—a percentage of 1.5%.

"As far as could be ascertained, the first patient has not been near any areas in which infection occurred in [illegible] nor could any person be found in her neighbourhood who had been in these districts—in other words no evidence whatever could be found to indicate contact with any of the areas where infection was known to have previously occurred.

"Following the discovery of the first case, 2 others were found. The second patient had vomiting and diarrhoea with fever but was well again in 4 days, while the third patient remained ill for several days."

The following remarks are quoted from the annual report for 1940 of the League of Nations Singapore Bureau.

"During the period 16 June to 27 July 1940 a further 8 cases of El Tor infection were notified from Celebes, of which 5 were fatal.

"Some interesting points in regard to this disease have been brought to notice. One of these is the high mortality, viz. 65%, which resembles that of true cholera. Another is that it does not tend to assume epidemic proportions; for example, there was only 1 case in each of 14 villages, 2 cases in each of four villages and 5 cases in one village only. In addition, with odd exceptions, there was never more than 1 case in a family."

It is relevant to state that in India the El Tor vibrio has repeatedly been found in water even in areas entirely free from cholera. The question whether this vibrio may be etiologically related in India to any choleraic disease, as was evidently the case in Celebes, has not yet been satisfactorily answered.

Singapore

Singapore Island has been free from the disease since the last outbreak in 1926-28 (see Table VI).

Japan

Table VI shows that prior to the Second World War the disease had almost completely disappeared from the Japanese islands, although early in the present century comparatively severe epidemics were not infrequent. These manifestations were held to have been invariably due to an importation of the infection. Generally cholera gained impetus in the large seaports and was then spread along the coast by small craft, whose movements were difficult to control. According to Takano and co-authors (1926) the disease

"is most frequently imported in the months of August and September and these are the months in which epidemics reach their peaks. The epidemic begins to subside gradually in October and November and practically ceases in December."

During May 1946, however, the arrival of repatriation ships from China and other countries of the Far East led to an importation of cholera

Cholera in Insular Countries of Asia

Philippines

The insular countries of the Far East have also remained fairly free from cholera in recent years. For instance, Table VI shows that since 1923 in the Philippines there have been only two epidemic waves, one reaching its peak in 1925 the second outbreak starting in the year 1930 and continuing for five successive years, i.e. until 1934 during which period the disease prevailed in the central, densely populated archipelago In November 1933 the disease was present on the west coast of Bohol Island and the opposite east coast of Cebu Island. While it disappeared from the latter island early in 1934 it spread to the east coast of Negros Island, but died out by April 1934

From 1938 onwards the islands have remained free from cholera.

Indonesia

The island of Java recorded its last major epidemic of cholera in 1918-19 Thereafter as shown in Table VI, only sporadic cases have occurred. Thus in the year 1927 there were 10 deaths, and in 1928 only one death from the disease The island has now been free from cholera for over a quarter of a century Even in earlier years following the First World War the infection, when imported was quickly exterminated.

An outbreak which aroused considerable epidemiological interest started in September 1937 on the island of Celebes, Indonesia. The first cases were notified from Pangkadjene which is situated about 40 miles north of Macassar The patients in question showed clinical symptoms of cholera, and the vibrios, isolated from the stools of some of them, were shown to be of the El Tor variety From 26 September to 11 December 1937 there were 17 cases and 11 deaths in this area. The disease did not manifest itself again until 9 January 1938 when there were 12 deaths up to 19 February The total number of cases from 26 September to 29 April 1938 was 35 27 of whom died. It was reported that from 31 August 1937 to 23 September 1937 already six people had died with symptoms suggesting cholera infection on the island of Samtolle north-west of Macassar Three further cases occurred on the same island towards the end of September two of whom were fatal From these patients also El Tor vibrios were isolated The epidemiological features of this outbreak and the characteristics of the vibrio isolated have been discussed by de Moor (1938)

A further instance of infection with the El Tor vibrio was observed in Celebes on 27 October 1939 the patient concerned dying within 24 hours after the appearance of symptoms. To quote from the annual report for 1939 of the League of Nations Singapore Bureau

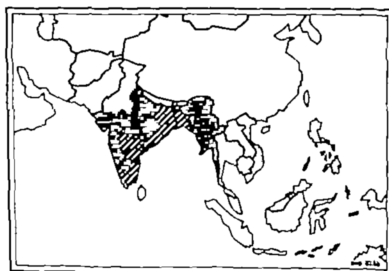
"In the area in which this first case occurred there were 20 further cases in the succeeding two months while further south 5 cases occurred in one week in a small

TABLE VIII CHOLERA DEATH RATE PER 100 000 POPULATION 1950-54

Country	Cholera death-rate mean	Cholera death-rate median	Country	Cholera death-rate mean	Cholera death-rate median
India			India (*continued*)		
Ajmer-Merwara	2.3	—	Madhya Pradesh	23.4	8.5
Assam	6.3	3.0	Uttar Pradesh	11.4	9.5
Bihar	32.0	34.8	West Bengal	31.5	24.4
Bombay	13.3	4.8	Pakistan		
Coorg	0.1	—	East Bengal	41.6	43.8
Delhi	0.3	—	West Pakistan	—	—
Hyderabad	37.3	23.9	Burma	7.9	1.3
Madras	28.6	27.7	Ceylon	0.0	—
Mysore	18.4	14.3	Cambodia	0.2	0.1
Orissa	28.7	28.6	Viet Nam	0.0	0.0
Punjab	0.3	0.1	Laos	0.3	—

This shows that India and Pakistan together constituted 98% and with Burma 99.98% of the total cholera deaths in the world during 1950 54 Within India and Pakistan the highest incidence of the disease is reported from its endemic home in the two Bengals (East and West) into which the

FIG 4 GEOGRAPHICAL DISTRIBUTION OF CHOLERA, 1950-54



Median death-rate per 100 000 inhabitants (1948-54)

- Less than 0.01
- 0.01-0.9
- 1.0-4.9
- 5.0-19.9
- 20.0 and over

through the port of Uraga. The epidemic soon spread and the incidence of the disease during the year reached a total of 1229 cases with 528 deaths. From 1947 onwards Japan has been completely free from cholera.

Taiwan

Although no complete figures for Taiwan are available, Table VI indicates an almost negligible incidence of the disease in the island from 1923 to 1943 Even the major epidemic taking place on the Chinese mainland in 1932 caused only minor repercussions in Taiwan For as reported by Shimoji and co-authors (1933) the importation of the infection in 1932 by the crew of a ship led to the appearance of only 17 manifest cases (with six deaths) and to a carrier state in six further apparently healthy persons Twenty out of these 23 infected individuals had attended the funeral of the captain of the junk and had consumed obviously cholera-contaminated food with the crew

However serious cholera outbreaks, due to an importation of the infection, took place in Taiwan in 1943 and in 1946

Ceylon

The history of cholera in Ceylon is that of repeated importations of the infection from India. As shown by a comparison of the series of annual figures for the two countries embodied in Table VI outbreaks in Ceylon occurred generally one year after a high incidence of the disease had been recorded in India.

The last major outbreak, resulting in 419 deaths, took place in Ceylon in 1919 when cholera showed a peak incidence in India. From 1947 onwards the incidence of the disease in Ceylon has been negligible

Cholera in the Asiatic Mainland

World cholera mortality rates per 100 000 population in the recent five year period 1950 54 are shown in Table VIII Because of their size, the figures for India and Pakistan are shown separately by individual States

The total deaths from cholera during 1950 54 constituted only about 0 1 % of the estimated deaths from all causes in the world

Fig. 4 showing the geographical distribution of the disease, indicates that in the recent post war years cholera has been confined mainly to the Asiatic mainland, largely to India, East Pakistan and Burma. The percentages of the world cholera deaths reported from these countries are

Country	%
India	75 48
Pakistan	22.57
Burma	1 93
	99 98

doubt that cholera has now become endemic in several parts of China" It is noteworthy, however that he qualified this statement by adding that "nevertheless, there is evidence to show that it is not endemic in many areas which have been visited by severe epidemics"

The numbers of cholera cases reported in the various provinces of China from 1939 to 1950 are shown in Table IX

Exact information regarding the number of cholera cases or deaths for an earlier period is not available, but an approximate idea of the incidence of the disease has been provided in Table VI

During the years 1949 50 the mainland of China appears to have been free from cholera, and, to judge from press reports becoming available in March 1957 the infection has continued to be absent This state of affairs supports the contention that cholera is not endemic in China.

The first epidemic occurring during 1923 54 arose in China in 1926 It is known that even during 1925 cholera was widespread in the country only Yun nan in the extreme south west having remained entirely free from the infection Nevertheless, during that year the disease was largely confined to the coastal plains north and south of the Yangtze as well as to the whole of the south coast. Having been prevalent in 1925 during the months of July and August, cholera appeared in mild form in the Provinces of Che kiang, An-hwei Honan and Shan si in the earlier part of 1926 In the months of July and August 1926 however epidemics involving all the coastal districts up to Manchuria appeared while in Shanghai the infection of the Chapei waterworks led to a serious situation The disease also spread to Korea in 1926 where 13 districts were reported to have become affected However owing to strict measures, each of these manifestations was quickly controlled so that only 159 deaths resulted.

In 1927 the disease was only moderately prevalent in Shanghai but there were serious epidemics in Kwang tung Province in the south and Chihli Province in the north During 1927 to 1931 although several areas continued to report cases, the incidence was much lower except that Shanghai had an epidemic in July 1929 which however, was less severe than that of 1926

In 1932, China suffered from a severe manifestation of cholera which could well be regarded as pandemic in character outbreaks occurring in all the eastern provinces in the north as well as the south A rough idea of the incidence of these epidemics and the extent of their spread is provided by the figures in Table X.

During 1931 in spite of disastrous floods in the Yangtze valley the cholera incidence in China remained very low only one death being recorded in Canton while the ports and provinces in the north remained free from the infection In March 1932, the disease first appeared in epidemic form at Canton then in April at Shanghai During May cholera spread to Swatow and inland to Han kow and Nanking, and by June it had also affected several

old presidency of Bengal was divided following the partition of the country in August 1947

An account of the recent occurrence of cholera in these and the other countries involved on the Asiatic mainland follows.

China

As summarized by Wu Lien-teh and co-authors (1934) 46 cholera manifestations of a more or less serious nature were recorded in China from 1817 to 1934 ten of which led to a spread of the infection as far north as Manchuria Following a serious and a widely disseminated outbreak in 1919 during the period under review cholera epidemics in China were recorded in 1926 1932, 1937 39 1942, and 1946 From 1937 onwards owing to war conditions, the disease showed a tendency to become more widespread and persistent than had been the case in the past However as will be shown later the period after 1946 is characterized by a most marked decrease of cholera in China and evidently its ultimate disappearance.

Reliable numerical information regarding the incidence of cholera in earlier years in China is not available because of the insufficient system of disease-reporting. It was only in 1926 that the National Epidemic Prevention Bureau at Peking inaugurated a system for the reporting on the epidemic situation Nevertheless, the large size of the country the inadequacy of health staffs, and civil wars rendered it impossible to obtain complete records The information available, therefore, indicates only the relative magnitude of the cholera prevalence from year to year and its seasonal variation. But no proper estimate of the total incidence can be made.

It has been shown by Swaroop & Pollitzer (1952) that during 1939-48 the area where cholera showed the greatest tendency to persistence lay in the delta of the Si-kiang River in the two southern provinces of Kwang tung and Kwang-si, possibly extending from the coastal and deltaic regions of Kwang tung westwards to Yun-nan and northwards along the coast into Fu kien Province. Some persistence of the infection to a comparatively minor degree has also been observed in Hu-nan in a locality on the Yuan River near Tung-ting Lake. The deltaic areas of the Yangtze river do not seem to have been as favourable for the persistence of the infection as the Si kiang Delta.

Ever since statistical data were collected for certain provinces of China, no year until 1947 was found free from the disease, which was thus constantly present in some part of the country Still, the absence of a really prolonged persistence of the infection in any of these areas renders it likely that most of the major epidemics in China were due to importations of the infection, if not from abroad, at least from areas within the country where a state of what might be called " temporary endemicity " had established itself It has to be mentioned, however that Stowman (1946) was not in agreement with this assumption maintaining that " there can be little

TABLE X CHOLERA EPIDEMIC IN CHINA DURING 1932

Region	Infected Cities	Cases	Deaths
Shanghai	1	4 760	317
Nanking	1	1 588	373
Peiping	1	493	391
Kiang su	38	10 430	1 606
Hopeh	64	14 617	5 036
Ho-nan	30	10 558	2 362
Shan-tung	27	18 153	2 962
Shan-si	29		6 926
Kiang si	14	5 918	1 955
An-hwei	19	3 349	1 214
Shen si	17	12 644	3 468
Hu-peh	12	2 832	1 231
Che-kiang	13	6 423	657
Suiyuan	6	2 000	1 057
Chahar	7	618	183
Fu-kien	5	1 679	973
Hu-nan	4	1 653	338
Kwang-tung	2	1 084	358
Yun-nan	3	54	9
Chinghai	3	121	12
Sze-Chwan	6	1 969	501
Kwang-si	1	2	1
Kan su	3	222	78
Total	306	100 666	31 974

From the Report of National Flood Relief Commission 1931-2, quoted by Wu Lien-teh and co-authors (1934).

Manchurian ports in the north (Antung, Newchwang, and Dairen) In July the infection had reached the north western provinces of Sui yüan Shen si and Hu nan The floods in Manchuria in August aggravated the epidemic situation The number of cases was estimated at more than 100 000 and that of deaths at 31 974 Although cholera infected boats are known to have carried the infection to Japan the general effect of the great epidemic in the countries outside China was that it appeared only in the form of short outbreaks with comparatively few victims

Cholera in China subsided during the year of 1933 when only a very low incidence was recorded Except a few isolated cases in the ports of Canton

TABLE IX. CHOLERA CASES REPORTED IN CHINA FROM 1939 TO 1950

Region	Population	1939	1940	1941	1942	1943	1944	1945	1946	1947	1948	1949	1950
Fu-kien	11 101	83	4 036	5	6	1 481	239	40	1 656	98	0	0	0
Taiwan	6 126	0	0	0	0	0	0	0	2 627	0	0	0	0
Kwang-tung	27 826	0	427	285	1 736	7 083	22	653	7 731	41	0	0	0
Kwang-si	14 603	626	18	0	6 621	2 639	393	1 948	1 992	0	0	0	0
Yun-nan	9 171	3 604	0	0	7 680	606	53	212	19	0	0	0	0
Kwei-chow	10 519	3 684	1	0	2 854	2 404	361	2 204	16	0	0	0	0
Kiang-su	36 052	0	0	0	0	0	0	18	6 336	724	1	0	0
Che-kiang	19 942	0	9 673	0	136	145	0	22	6 902	309	0	0	0
An-hwei	21 705	0	0	0	0	0	0	0	3 092	1	0	0	0
Kiang-si	12 725	5 020	17		601	433	7	0	1 231	3	9	0	0
Hu-peh	21 034	4 664	3	0	164	0	42	265	671	0	2	0	0
Hu-nan	28 171	1 904	103	79	4 455	2 357	26	1 300	3 159	26	0	0	0
Sze-Chwan	47 108	4 051	145	0	394	23	27	10 010	115	8	0	0	0
Sikong	1 651	118	0	0	0	0	0	403	0	4	0	0	0
Ho-peh	28 629	0	0	0	0	0	0	0	649	0	0	0	0
Shan-tung	38 672	0	0	0	0	0	0	0	396	0	0	0	0
Shan-si	15 025	49	0	0	0	0	0	0	13	0	0	0	0
Ho-nan	28 473	361	117	0	0	0	2	5	2 064	1 326	0	0	0
Shen-si	9 492	10 030	0	0	0	0	0	167	0	0	0	0	0
Kan-su	6 898	15	0	0	0	0	0	114	0	0	0	0	0
Ningsia	738	0	0	0	0	0	0	0	40	0	0	0	0
Liao-ning	9 992	0	0	0	0	0	0	0	3 324	0	0	0	0
Liao-peh	3 798	0	0	0	0	0	0	0	2 625	0	0	0	0
Kirin	6 981	0	0	0	0	0	0	0	4 954	0	0	0	0
Jehol	6 110	0	0	0	0	0	0	0	194	0	0	0	0
Sinkiang	4 012	0	0	0	0	0	0	0	27	0	0	0	0

In thousands

Even though August was usually the month of highest cholera incidence, the generally mild but most extensive outbreak taking place in China during 1946 gained momentum and started to spread in July By October 1946 the disease had reached as far north as central Manchuria and Inner Mongolia as far south as Hoppo (Lim-chow) east of the Tong-king border as well as 100 miles west of Chung king

Thus, while the epidemics of 1926 1932, and 1942 had affected chiefly the lower and middle Yangtze areas, and the 1937 39 outbreaks had been most severe farther west, the 1946 epidemics extended to the utmost confines of the country There can be no doubt that this exceedingly wide spread was due to the return of displaced persons as well as to troop movements and the repatriation of Japanese internees

The ravages of the 1946 outbreaks were worst, by far, in Manchuria, where the disease had been absent for many years Probably imported by troops coming from south China, cholera appeared in Manchuria in the second half of June at Liao-ning. An explosive spread of the infection followed, and the area outside the Communist zone was affected within 2 3 weeks, the infection mainly spreading along the railway lines. Though, generally information is available only for cities and communities along the railway lines, there can be no doubt that in 1946 cholera exacted a death toll in Manchuria which equalled or even surpassed that caused in this part of China by the previous most severe visitations of the disease

Though there were probably also some smaller foci where the infection had persisted during the winter of 1945-46 to become recrudescent in spring it appears that there were two main foci from where the 1946 epidemic originated—the one in Kwang tung, the other on the middle Yangtze. The outbreaks traceable to these main foci though becoming to some extent superimposed, remained distinct in their behaviour The Yangtze epidemics were characterized by a low or moderate case fatality rate (approximately 10%) while in case of the outbreaks derived from Kwang tung the case fatality was as high as 25% to 30%

Thailand

The trend of cholera in Thailand from 1917 to 1954 is illustrated in Fig. 5 During the last few years this disease has been almost absent from the country for while in 1948 it still caused 15 deaths, only one cholera fatality was recorded in 1949 and none during the years 1950-54 A solitary cholera case was observed in the Province of Prachinbury in 1951

In marked contrast to India East Pakistan and Burma with a persistently high cholera incidence Thailand presents features characteristic of an area liable only to epidemic inroads of the infection For as shown in Table VI epidemic periods with a high cholera mortality such as those of 1925-29 1935-37 and 1943-47 lasting 3 5 years, alternated with interepidemic

Shanghai, and Han-kow the year 1934 also remained almost free from the disease

In May 1937 cholera once more broke out in epidemic form in the eastern part of Kwang tung, and by August had reached Kwang si. It is estimated that over 28 000 cases occurred in the former province but only 600 in the latter By December of 1937 the infection became manifest in the Yuan River basin and in June of the following year three other river systems, namely those of the Tse the Siang, and the Yangtze, were found to have become involved. The epidemic had thus spread as far north as Hu nan where over 4500 cases with more than 2000 deaths occurred.

In 1939 another serious outbreak took place in the Sze Chwan and Shen-si Provinces where the two major cities of Chung king and Cheng-tu became distributing centres of the infection. This outbreak is believed to have originated towards the end of June in the hilly rural regions of Nangpu Hsien (district) situated on a tributary of the Kialing River from where it spread to a number of neighbouring districts to the west and the north west, causing over 41000 deaths in Sze-Chwan and more than 10000 in Shen-si While only 470 cases were reported in Hu nan to the south, Hu peh and Kiang si Provinces became seriously involved Besides thus reaching its highest incidence in central China, cholera also spread into south western China However, by the end of August the epidemic had practically subsided.

The low incidence in the interior of China during 1941 is of interest in view of the fact that cholera was rampant in that year in the port cities of Macao (1475 cases) Hong Kong, Canton, and Shanghai This led Stowman (1945) to observe that " it is clear that cholera epidemics in the interior do not necessarily follow upon high prevalence in the ports "

The 1942 epidemic was mainly confined to south China, where the infection continued to exist, leading to an outbreak in Kwang tung in September 1945 and to a recrudescence of this epidemic in May of 1946 The appearance of cholera in Hu nan in 1945 probably stood in causal connexion with its presence in Kwang tung.

During 1945 the disease was also present in mild form in Kwang-si, Kwei-chow and Yun nan The extensive outbreak taking place in that year in Sze-Chwan started in May on the Yangtze above Chung-king. This city and its neighbourhood became affected early in June. An extension of the infection down the Yangtze River as far as Ichang followed and, while the epidemic in Sze-Chwan had subsided by the end of 1945 cholera continued to exist with moderate intensity at Ichang throughout the winter This presence of the disease at Ichang seems to have been responsible for a severe outbreak among the Japanese concentrated in a camp at Han kow during February 1946 Being afterwards transported in barges from Han kow the former inmates of this camp carried the infection to Nan-king and Shanghai.

May 1945 to be "spreading seriously" in the Mekong Delta and to be descending along the Menam towards Bangkok As shown in Table VI, the infection continued to be present in Indochina even in 1948 whereas, as noted above the last epidemic period in Thailand, which had commenced in 1943 terminated one year earlier, in 1947

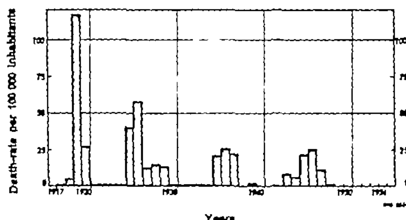
Table XI shows that the cholera affected areas in Indochina are usually those in the south i e Cochin China and Cambodia, rather than the

TABLE XI. CHOLERA CASES AND DEATHS IN INDOCHINA FROM 1922 TO 1954

Year	Annam		Cochin-China		Cambodia		Laos		Tong king	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
1922	255	124	934	632	382	221	89	15	0	0
1923	43	17	199	11	40	10	4	0	39	4
1924	16	?	55	28	49	28	0	0	33	15
1925	48	6	66	39	91	43	1	0	26	8
1926	1 315	974	5 035	3 979	5 625	4 767	586	556	6 478	4 149
1927	5 287	4 024	2 107	1 422	825	610	201	113	21 652	18 032
1928	577	349	4 187	3 522	1 362	1 015	0	0	63	19
1929	164	71	3 201	2 726	1 746	1 144	28	11	9	3
1930	18	12	1 954	1 544	1 312	847	28	3	1	0
1931	20	11	866	633	1 381	938	26	0	0	0
1932	65	33	260	194	199	133	7	3	0	0
1933	2	1	126	99	152	90	0	0	0	0
1934	1	1	82	67	38	26	0	0	0	0
1935	3	2	54	40	68	44	0	0	0	0
1936	0	0	36	34	33	27	0	0	0	0
1937	1 940	1 462	4	4	13	11	0	0	10 271	7 415
1938	3 540	2 554	0	0	0	0	0	0	4 967	4 072
1939	0	0	0	0	0	0	0	0	1	1
1940-46										
1947			522	363	2 300	1 681	104	64		
1948			596	384	1 526	1 193	0	0		
1949			9	6	21	14	0	0		
1950			13	3	5	3	0	0		
1951			18	14	63	5	0	0		
1952			7	6	26	11	0	0		
1953			3	3	4	4	31	18		
1954			0	0	8	4	0	0		

* From 1947 the figures relate to Viet Nam as a whole.
** From 1947 the figures include suspected cases.

FIG 5. CHOLERA MORTALITY IN THAILAND 1917-54



periods of a similar length during which the incidence of the disease was low or even insignificant

Although cholera is apparently not endemic in Thailand, its importation has led in the past to violent and prolonged epidemics. For instance, the arrival in Bangkok, in October 1925 of a cholera-stricken steamer from Swatow led to an outbreak lasting from November to December in the former port, which caused over 3000 cases with more than 2000 deaths After a temporary seasonal decrease in January and February 1926 the infection flared up once more and, leading to a peak incidence in May continued to be manifest until the end of October This epidemic reached French Indochina through Cambodia in January 1926

The 1935 epidemic was presumably imported from Burma

The last epidemic, which terminated in 1947 was also of prolonged duration being spread over a period of five years River and canal water pollution is believed to be the greatest source of danger in the dissemination of cholera in Thailand, the disease being prevalent for longer periods in the southern provinces along the river Chao Phya

Indochina (Cambodia Laos Viet Nam)

Like Thailand, Indochina is an area liable to cholera epidemics of a highly explosive nature.

A comparison of the series of figures for these two countries (Table VI) shows that in Indochina the cholera epidemics have a tendency to break out one year later than in Thailand and to last somewhat longer For instance, the epidemic outbreak of 1925 in Thailand led to an importation of the infection into Indochina, which resulted in an explosive outbreak first in Cambodia in January 1926 While in Thailand this epidemic came to an end during 1929 the incidence of cholera in Indochina continued to be high for another two years.

According to Stowman (1945) Indochina was relatively free from cholera during the war period of 1941-44 but the disease was reported in

May 1945 to be "spreading seriously" in the Mekong Delta and to be descending along the Menam towards Bangkok. As shown in Table VI the infection continued to be present in Indochina even in 1948 whereas as noted above, the last epidemic period in Thailand which had commenced in 1943 terminated one year earlier in 1947.

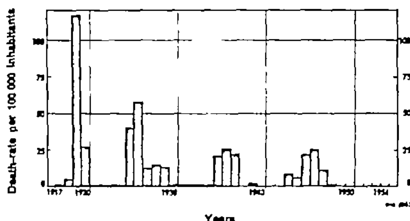
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TABLE XI CHOLERA CASES AND DEATHS IN INDOCHINA FROM 1922 TO 1954

Year	Annam		Cochin China		Cambodia		Laos		Tongking	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
1922	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	15	0	0
1923	43	17	[illegible]	11	[illegible]	[illegible]	4	0	[illegible]	4
1924	10			[illegible]	[illegible]	[illegible]	[illegible]	0	33	1
1925	4	[illegible]	[illegible]	[illegible]	91	43	1	0	26	8
1926	[illegible]	974	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	6 478	4 149
1927	[illegible]	4 024	[illegible]	[illegible]	[illegible]	610	[illegible]	113	21 652	18 032
1928	[illegible]	349	[illegible]	[illegible]	[illegible]	[illegible]	0	0	63	19
1929	104	71	[illegible]	[illegible]	1 740	1 144	[illegible]	11	9	3
1930	18	1	1 954	1 644	[illegible]	647	[illegible]	3	1	0
1931	[illegible]	11	[illegible]	633	1 331	933	[illegible]	0	0	0
1932	65	33	[illegible]	194	199	133	7	3	0	0
1933	2	1	176	99	[illegible]	90	0	0	0	0
1934	1	1	82	67	38	26	0	0	0	0
1935	3	2	[illegible]	40	68	44	0	0	0	0
1936	0	0	36	34	39	27	0	0	0	0
1937	1 940	1 452	4	4	13	11	0	0	10 271	7 415
1938	3 540	[illegible]	0	0	0	0	0	0	4 967	4 072
1939	0	0	0	0	0	0	0	0	1	1
1940-46										
1947			522	363	2 300	1 681	104	64		
1948			696	384	1 626	1 193	0	0		
1949			9	6	21	14	0	0		
1950			13	3	5	3	0	0		
1951			18	14	53	6	0	0		
1952			7	6	26	11	0	0		
1953			3	3	4	4	31	18		
1954			0	0	8	4	0	0		

* From 1947 the figures relate to Viet Nam as a whole.
** From 1947 the figures include suspected cases

FIG 5. CHOLERA MORTALITY IN THAILAND 1917-54



periods of a similar length, during which the incidence of the disease was low or even insignificant.

Although cholera is apparently not endemic in Thailand, its importation has led in the past to violent and prolonged epidemics. For instance, the arrival in Bangkok, in October 1925, of a cholera-stricken steamer from Swatow led to an outbreak lasting from November to December in the former port, which caused over 3000 cases with more than 2000 deaths. After a temporary seasonal decrease in January and February 1926, the infection flared up once more and, leading to a peak incidence in May, continued to be manifest until the end of October. This epidemic reached French Indochina through Cambodia in January 1926.

The 1935 epidemic was presumably imported from Burma.

The last epidemic, which terminated in 1947, was also of prolonged duration, being spread over a period of five years. River and canal water pollution is believed to be the greatest source of danger in the dissemination of cholera in Thailand, the disease being prevalent for longer periods in the southern provinces along the river Chao Phya.

Indochina (Cambodia, Laos, Viet Nam)

Like Thailand, Indochina is an area liable to cholera epidemics of a highly explosive nature.

A comparison of the series of figures for these two countries (Table VI) shows that in Indochina the cholera epidemics have a tendency to break out one year later than in Thailand and to last somewhat longer. For instance, the epidemic outbreak of 1925 in Thailand led to an importation of the infection into Indochina, which resulted in an explosive outbreak first in Cambodia in January 1926. While in Thailand this epidemic came to an end during 1929, the incidence of cholera in Indochina continued to be high for another two years.

According to Stowman (1945), Indochina was relatively free from cholera during the war period of 1941-44, but the disease was reported in

May 1945 to be "spreading seriously" in the Mekong Delta and to be descending along the Menam towards Bangkok As shown in Table VI the infection continued to be present in Indochina even in 1948 whereas, as noted above the last epidemic period in Thailand which had commenced in 1943 terminated one year earlier, in 1947

Table XI shows that the cholera affected areas in Indochina are usually those in the south i e Cochin China and Cambodia, rather than the

TABLE XI CHOLERA CASES AND DEATHS IN INDOCHINA FROM 1922 TO 1954

Year	Annam		Cochin-China		Cambodia		Laos		Tong King	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
1922	255	1 4	964	632	382	221	89	15	0	0
1923	43	17	199	112	40	10	4	0	39	4
1924	16	2	55	28	49	28	0	0	33	15
1925	48	6	66	39	91	43	1	0	26	8
1926	1 315	974	5 035	3 979	5 625	4 767	586	556	6 478	4 149
1927	5 287	4 024	2 107	1 422	825	610	201	113	21 662	18 032
1928	577	349	4 187	3 522	1 362	1 015	0	0	63	19
1929	164	71	3 201	2 776	1 746	1 144	28	11	9	3
1930	18	12	1 954	1 544	1 312	847	28	3	1	0
1931	20	11	866	633	1 381	938	26	0	0	0
1932	65	33	260	194	199	133	7	3	0	0
1933	2	1	126	99	152	90	0	0	0	0
1934	1	1	82	67	36	26	0	0	0	0
1935	3	[illegible]	54	40	68	44	0	0	0	0
1936	0	0	36	34	38	27	0	0	0	0
1937	1 940	1 462	4	4	13	11	0	0	10 271	7 415
1938	3 540	2 654	0	0	0	0	0	0	4 967	4 072
1939	0	0	0	0	0	0	0	0	1	1
1940-46										
1947			522	363	2 300	1 681	104	64		
1948			596	384	1 626	1 193	0	0		
1949			9	6	21	14	0	0		
1950			13	3	6	3	0	0		
1951			18	14	53	5	0	0		
1952			7	6	26	11	0	0		
1953			3	3	4	4	31	18		
1954			0	0	8	4	0	0		

* From 1947 the figures relate to Viet Nam as a whole.
From 1947 the figures include suspected cases.

central and northern part of the country comprising Annam, Laos and Tong-king. The 1937 38 epidemic was an exception since, being connected with an influx of refugees from south China, it was most prevalent in Tong king and Annam

Burma

Although Burma is geographically contiguous to the endemic home of cholera in Bengal it is virtually cut off from India by the land route, there being no interconnecting railway communication or any major highway The sea traffic from the highly infected port of Calcutta to the ports in south Burma is the connecting link and it is believed to have been responsible for keeping the incidence of cholera high in this country Indeed, as shown in Table VI since 1900 not a single year has passed in which cholera deaths did not occur in Burma. The last major epidemics occurred in the years 1915 and 1919 Thereafter outbreaks of lesser severity were recorded during 1926-29 and in 1932, 1935 1937 1940 1945-46, and 1950-51 Owing to the Japanese occupation of the country figures for the years 1941-44 are not available.

While Upper Burma is hilly and sparsely populated the delta of the Irrawaddy River in Lower Burma is low lying and much more populated It is, therefore not surprising to find that, as stated by Norman White (1923) the incidence of the disease is always higher in Lower Burma than in Upper Burma The delta region of Lower Burma usually suffers most and it is from this area that epidemics appeared to spread

Although health authorities are often prone to ascribe cholera epidemics to an importation of infection from the neighbouring countries, this has generally not been pleaded in the case of Burma, in spite of the fact that the country lies in close proximity to a principal focus of the disease in Bengal. The Director of Public Health Burma, for instance, admitted in his report for the year 1937 that cholera *has* been endemic in the Myaungmya district situated in the delta region of the Irrawaddy Reference to this district is also made in the following description of the 1934 cholera outbreak which may be considered as typical for the distribution and spread of the disease in Lower Burma

> "Starting in the Myaungmya district in October cholera spread to the adjacent districts of Ma-ubin and Pyapon. Bassein became involved in November These four districts, situated in the delta, are characterized by a network of waterways, with a large proportion of the population living and moving about in boats. The river in many cases, fulfils the threefold function of a water supply a washing place, and a latrine. Once cholera broke out, everything favoured its spread and, in a short time, cases were occurring simultaneously in every part of the affected district."

Swaroop & Pollitzer (1952) concluded from a study of the cholera mortality figures for individual districts during the period 1918-38 that a focus of cholera endemicity was situated in Burma within the Irrawaddy

delta in the three low lying districts of Myaungmya, Pyapon and Ma ubin The adjoining districts of Rangoon, Hanthawaddy, Insein and Bassein also showed a comparatively longer persistence of the disease than the remaining northern districts of Burma A comparatively minor degree of persistence was noted also in the Thaton districts in the delta of the Salween River

In Lower Burma, the disease has a tendency to reach its peak during the months of April May, and June, this period of highest incidence being followed by a decline during the monsoon season and the winter months The seasonal incidence of cholera in Upper Burma is different, however, the peak incidence being reached during the months of August, September, and October while a relatively low incidence of the disease is noted during the first six months of each year The unfortunate result of this seasonal difference is that, once cholera has broken out in Lower Burma the infection is apt to reach Upper Burma during a season favourable to the epidemic spread of the disease

Pakistan

For purposes of securing comparability in the series of cholera figures for India given from 1900 onwards, cholera deaths occurring in what is now Pakistan are also shown in the total for India in Table VI Separate figures of cholera deaths in Pakistan during recent years are shown in Table XII for each province and for the whole country

TABLE XII ANNUAL CHOLERA DEATHS BY PROVINCES IN PAKISTAN 1947-54

Year	East Bengal	West Punjab	Baluchistan	North-West Frontier Province	Sind	Total
1947	24 825	3 442	—	10	380	28 657
1948	29 691	2 222	—	136	1	32 050
1949	20 942	140	—	2	—	21 084
1950	23 911	—	—	—	—	23 911
1951	17 314	—	—	—	—	17 314
1952	19 416	—	—	—	—	19 416
1953	18 424	—	—	—	—	18 424
1954	8 423	—	—	—	—	8 423

Preliminary figures.

Consequent to the large-scale movement of refugees between India and Pakistan after the partition in August 1947 cholera broke out in the Punjab and spread to the North West Frontier Province and Sind However apart from this unusual occurrence, cholera in Pakistan has been confined to East Bengal which happens to lie in the endemic zone of this disease

central and northern part of the country comprising Annam, Laos and Tong-king. The 1937 38 epidemic was an exception, since being connected with an influx of refugees from south China, it was most prevalent in Tong king and Annam.

Burma

Although Burma is geographically contiguous to the endemic home of cholera in Bengal it is virtually cut off from India by the land route, there being no interconnecting railway communication or any major highway The sea traffic from the highly infected port of Calcutta to the ports in south Burma is the connecting link, and it is believed to have been respon sible for keeping the incidence of cholera high in this country Indeed as shown in Table VI since 1900 not a single year has passed in which cholera deaths did not occur in Burma. The last major epidemics occurred in the years 1915 and 1919 Thereafter outbreaks of lesser severity were recorded during 1926-29 and in 1932, 1935 1937 1940 1945-46 and 1950-51 Owing to the Japanese occupation of the country figures for the years 1941-44 are not available

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"Starting in the Myaungmya district in October cholera spread to the adjacent districts of Ma-ubin and Pyapon. Bassein became involved in November These four districts, situated in the delta, are characterized by a network of waterways, with a large proportion of the population living and moving about in boats. The river in many cases, fulfils the threefold function of a water supply a washing place, and a latrine. Once cholera broke out, everything favoured its spread and, in a short time, cases were occurring simultaneously in every part of the affected district."

Swaroop & Pollitzer (1952) concluded from a study of the cholera mortality figures for individual districts during the period 1918-38 that a focus of cholera endemicity was situated in Burma within the Irrawaddy

At first glance, a scrutiny of Fig 6 seems to provide indications of some long term decrease of the cholera incidence in India In fact, some of the earlier workers who did not have access to a sufficiently long series of figures, were tempted to conclude that the incidence of the disease was sharply decreasing. Indeed such a conclusion would be well nigh inevitable if one were to chart for example, only the figures from 1900 onwards and, more still, if studies in this direction had been made prior to the 1941-45 epidemic. For then starting from the highest peak each successive epidemic up to 1938 would have appeared to be reduced in magnitude However, the high peak attained by the epidemic in 1943 and its continuance through a 5-year period leaves room for doubt as to whether conditions have improved enough to bring the cholera epidemics under control Probably the only conclusion which it seems legitimate to draw is that after the 1919 epidemic the cholera incidence during interepidemic periods has been of a low order in India It has to be noted in this connexion that some time after the year 1923 public health services were established or expanded in various parts of India, the efforts of which were at first largely devoted to the control of epidemic diseases. It is also important to note that Fig. 6 is based on the total cholera mortality recorded throughout the Indian sub-continent, while the individual provinces show considerable heterogeneity regarding the length and severity of their epidemic outbreaks Further Fig 6 does not provide any indication of a regular periodicity in the incidence of cholera, since—as stated already—a search for periodicity has to be based on figures for areas homogeneous in regard to the epidemiology of the disease.

The peak incidence of cholera in some provinces is no doubt, partly explained by the holding of large scale pilgrim festivals, as, for example the Kumbh fairs taking place every six years at Hardwar situated in north west India in Uttar Pradesh (formerly United Provinces) and similar fairs held at different six yearly intervals at Allahabad in the east of that State Hardwar and Allahabad are the two most important pilgrim centres attracting devotees from all over the country Although Kumbh festivals are held at intervals of six years alternate festivals, celebrated every twelfth year have a greater religious significance However apart from these occasions Hardwar is visited by a stream of pilgrims from different parts of India almost throughout the year

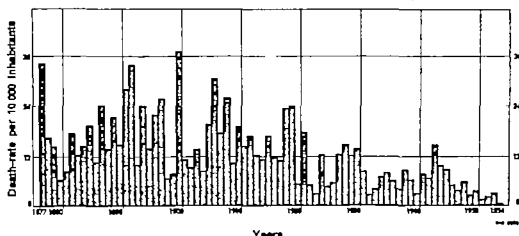
The close association between the Kumbh fairs and cholera incidence is amply shown by the history of cholera in the United Provinces and the adjoining province of the Punjab which lies farther west. Proof for this contention is furnished by Fig. 7 in which the bars showing the cholera mortality for each year since 1877 have been shaded in different ways so as to indicate the years in which full or half (Ardh) Kumbh fairs have been held at Hardwar or Allahabad As will be seen the majority of the years recording increases in cholera mortality were those in which fairs were held in the

The port of Chittagong, situated in this region, has reported cholera deaths annually since 1924 and shows (as indicated in Table XVII) a relatively higher degree of endemicity than any seaport in the East or in Far Eastern countries except Calcutta and Negapatam.

India

The annual cholera mortality rates per 10 000 inhabitants for what was formerly British India (excluding Burma) from 1877 to 1954 are shown in Fig. 6 For purposes of comparison the cholera deaths reported in Pakistan after the partition year of 1947 have been included in the corresponding rates However separate mortality figures for Pakistan have been given in Table XII

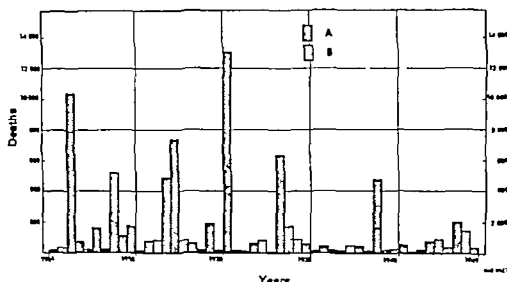
FIG 6. ANNUAL CHOLERA DEATH-RATE IN INDIA, 1877-1954



In India cholera has shown a markedly varying incidence from year to year with a maximum of 805 698 deaths in 1900 and a minimum of about 60 000 deaths in the more recent years up to 1953 Earlier as few as 66 137 deaths had been recorded in 1932. The last major epidemic occurring on the sub-continent reached peak incidence in the year 1943 with 459 930 deaths in what was then British India alone (excluding the States under local rulers) As will be seen later this unusual rise was attributable in a large measure to severe famine conditions in Bengal food scarcity in other areas and other shortcomings caused by the Second World War

Although in many cases in the past major outbreaks have been confined to a single year there have been instances when the cholera incidence remained at a high epidemic level for several years in succession. For instance, the epidemic starting in 1875 may be considered to have continued throughout a period of five years until 1879 Similarly the epidemic of 1927 although not attaining a very high peak, lasted until 1931 The last epidemic which, as noted above, reached its acme in 1943 may be said to have lasted from 1941 until 1945

FIG 8. ANNUAL CHOLERA DEATHS IN THE PUNJAB 1901-49 AND SOURCES OF INFECTION



A. Importation directly traceable to Hardwar United Provinces
B. Other sources of infection

concluded that almost invariably the manifestations of the disease there were the result of the Kumbh fairs held every six years in Hardwar

In regard to the second important pilgrim centre in Uttar Pradesh, it was stated by Lal (1937) that

> " At Allahabad each and every gathering at the time of the Kumbh and Ardh-Khumb from 1882 to 1918 was accompanied by a great rise of cholera incidence in the eastern districts of the United Provinces, Bihar and Orissa and the Central Provinces "

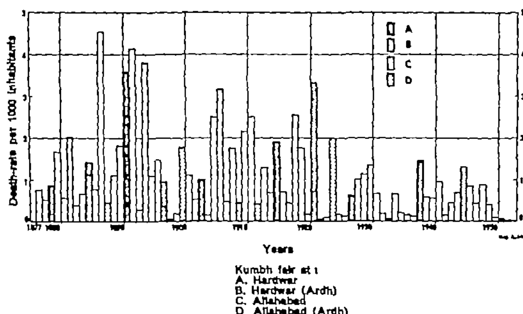
Lal added

> " The Ratha Yatra fair at Puri (Orissa State) Sinhast fair at Nasik (Bombay State) and Godavari Pushkaram fair at Rajamundry Krishna Pushkaram at Bezwada, Mahakam fair at Kumbakonam (Madras State) and Sagor mela at the Sundarbans (Bengal) occupy no less enviable positions in relation to cholera epidemics. Some of the epidemics in connexion with these fairs have led to world-wide epidemics."

One might, therefore be led to believe that in addition to Uttar Pradesh the States of Orissa, Bombay Madras and Bengal where the above mentioned pilgrim centres are situated would be particularly liable to suffer from cholera. For the sake of brevity it is not possible to discuss in detail the part these pilgrim centres have played in propagating epidemics Sufficient evidence has been quoted however to show that, in any study on the periodicity of cholera in India, due attention should be paid to the occurrence of important religious festivals and the holding of large scale congregations in general

There can be no doubt that the bulk of the reported cholera deaths in India occurred in what has been called above the major endemic home of the disease, namely in the provinces of Bengal Bihar Orissa, Assam and

FIG. 7 ANNUAL CHOLERA DEATH-RATE IN THE UNITED PROVINCES AND INFLUENCE OF KUMBH FAIRS 1877 1952



province though in some cases the incidence showed an increase in the year following that in which the fair was held, as for instance in 1880 1892, 1910 and 1913 There have been however striking exceptions, as for example in the years 1933 and 1942 during both of which fairs were held and for which only negligible cholera incidence was reported Such exceptions did occur even earlier as for instance in 1888 i e. at a time when cholera control measures during the fairs were not well developed or even altogether non-existent. It is also worth recording that in some years, as for instance in 1887 and 1908 the cholera mortality in the province was unusually high in spite of the fact that no large scale religious congregations had taken place While, therefore Fig. 7 is helpful in illustrating the important role of religious assemblies in giving rise to a high cholera mortality it also indicates that not necessarily each of these rises must have been the result of situations developing within the United Provinces—the less so because, as noted before a major cholera-endemic zone is situated to their east.

The association between Kumbh fairs at Hardwar and the incidence of cholera is more clearly evident in the case of the Punjab than in Uttar Pradesh. As will be gathered from Fig. 8 in which the annual cholera incidence in the Punjab from 1901 onwards is shown and where the various bars representing annual deaths are shaded in various ways to indicate the source of the infection, almost all major epidemics in the Punjab were attributable directly to an importation of the disease from Hardwar An identical opinion was reached by Yacob (1944) who tracing the origin of cholera outbreaks in the Punjab during the 77 year period of 1867 to 1943

epidemic proportions only during the monsoon months. The seasonal pattern of cholera in India indeed presents interesting features In Bengal, as already shown in Fig. 2 (page 55) cholera begins to rise in October and November and continues its upward trend to December and January Thereafter a decline occurs in February, followed by another rise until the peak is reached in April and May Although this seasonal pattern holds true for a large part of cholera-endemic Bengal a more detailed examina tion, as, for instance, that made by Lal and co-authors (1941), has demonstrated that conditions may differ markedly in different parts of the province. It has already been stated that, as the western borders of Bengal are reached, a curious variation is observed in the months of the south west monsoons In Madras Presidency which is affected by the south west monsoons in June, July and August and by the north-eastern monsoons along the Coromandel coast during the winter months of November and December two peaks occur synchronizing with the two monsoon periods of the year Almost all epidemics have adhered to this " time table " which in spite of more rapid means of transport in recent years and greater traffic from Bengal north-westwards, has not been altered possibly because of the consistency of the climatic factors. The presence of two seasons favourable each year to the epidemic spread of cholera probably accounts for the comparatively considerable and persistent incidence of the disease in Madras

During the period covered by this review cholera first showed an epi demic increase in 1924 simultaneously in Bengal, Bihar Orissa, Assam, and the United Provinces as early as March April, that is corresponding to the first seasonal peak of the year In the United Provinces the first cases of cholera occurred in February resulting in a violent epidemic at the end of March, which continued through the month of April and was on the decrease from May to July The onset of monsoon rains in August again increased the incidence both there and in Bihar and Orissa The wave of infection moved westwards into the Punjab in July but it did not progress farther north to the North West Frontier Province. The highest mortality, as usual was recorded in the middle and upper Ganges valley as well as in Assam No spread of the infection westwards from India is known to have taken place in 1924 while in the east, only Burma became invaded during that year With the exception of Korea the other still cholera-affected countries recorded a lesser incidence of the disease in 1924 than during the previous year

In the following two years i e 1925 and 1926 the disease was compara tively quiescent in its main centres in India although in the meantime it is known to have spread in epidemic form to Thailand and Indochina It has to be noted however that the comparative quiescence of cholera in India during 1925 was largely confined to its endemic home in Bengal and to the Indo-Gangetic plains while at the same time the disease was

the United Provinces. Table XIII which compares the cholera mortality by decades from 1910 onwards in Bengal Bihar and Orissa United Provinces, and Madras Presidency respectively with the corresponding figures for British India as a whole illustrates this contention Out of about 10 million cholera deaths which occurred in British India during this period, 29% were contributed by the Bengal Presidency the population of which was 21% that of British India. Another 25% occurred in Bihar and Orissa the population of which was 16% of that of British India. The United Provinces and Madras contributed 17% and 14% respectively the corresponding population percentages being 19% and 16%. Cholera deaths in these four provinces therefore, were responsible for 85% of the total cholera deaths in British India during the 45-year period of 1910-54

TABLE XIII CHOLERA DEATHS BY PROVINCES AND PERCENTAGE OF THE PROVINCIAL CHOLERA DEATHS TO TOTAL CHOLERA DEATHS IN BRITISH INDIA, 1910-54

Period	Deaths					Percentage of the total deaths in British India			
	Bengal	Bihar and Orissa	United Provinces	Madras Presidency	British India	Bengal	Bihar and Orissa	United Provinces	Madras Presidency
1910-19	1 004 624	993 344	678 269	616 738	3 808 006	26	26	18	16
1920-29	714 649	604 276	366 609	319 924	2 224 755	32	23	17	14
1930-39	520 666	421 608	258 108	160 488	1 692 001	31	25	16	11
1940-49	683 836	597 319	363 279	264 248	2 110 624	28	28	18	13
1950-54	128 521	83 867	36 165	82 088	380 110	33	22	10	22
1910-54	2 950 185	2 600 433	1 722 330	1 462 464	10 215 496 *	29	25	17	14

* Excluding figures for East Bengal for the year 1948.

When studying the occurrence of major cholera outbreaks in different parts of India, due attention should be paid to the marked differences in the seasons during which the disease is apt to become epidemic in different parts of the country For instance even though cholera is believed to be highly endemic in Bengal its incidence falls to a minimum during the monsoon months of June, July and August. Then as stated by Fry (1925)

"year after year as soon as the land dries up cholera reappears in mild epidemic form. If there have been tornados and tidal waves bringing salt water into the rivers and tanks, then as in 1876 and 1897 the cholera assumes fulminant epidemic proportions."

The dry season is also of epidemiological importance in so far as it is the season of movement, and there is much emigration and immigration of hired labourers for cutting the winter rice-crop Nevertheless, in certain non-endemic areas, e g., in the Punjab the disease has a tendency to reach

A sharp increase in the incidence of cholera occurring in 1938 was caused by a wide spread of the infection as the result of an outbreak taking place at the time of the Kumbh fair at Hardwar in April of that year The consequences of this initial epidemic for the Punjab are shown in Fig. 8 (page 81)

Although the Hardwar festival started on 1 February and lasted till the end of April the epidemic did not break out until a favourable season had set in That the progress of the disease from Hardwar northwards was extremely rapid is shown by the fact that, while in the Punjab the first case occurred during the week ending 9 April, in the following week 11 districts up to Lahore had been infected One week later another 16 districts had become involved and in the following week the North West Frontier Province had been reached

The United Provinces Bihar and Orissa, as well as the Central Provinces also suffered much The first mentioned area, bearing the brunt of this epidemic, recorded 70 622 deaths as against only 6341 deaths in 1937 In Bengal more than twice as many victims were recorded in 1938 as in during the previous year (71 133 deaths as against 32 700 deaths in 1937) However according to the Public Health Commissioner with the Government of India, this increase was apparently not associated with the Hardwar festival but was due to the occurrence of extensive floods in October 1938 the spread of the disease becoming considerable in the succeeding month of November The Central Provinces were also severely affected, the cholera mortality there during 1938 reaching a total of 45 332 as against only 1107 deaths in 1937

The high incidence of cholera during the period 1941 to 1945 in the course of which an unusually high peak was observed in 1943 was probably largely due to wartime conditions which began to exert an influence in India from 1941 onwards Serious shortages occurred not only in foodstuffs but also in respect of civil medical officers who had joined the army in large numbers, as well as in regard to medical and sanitary stores for the procurement of which the army had been given priority Additional hardship was caused by the invasion of the eastern frontier regions of India by Japanese forces and still more by the cutting-off of the large rice imports which in normal times used to come to India from Burma and other eastern countries It was under these conditions that a severe famine occurred in Bengal during which, according to conservative estimates over one and a half million people died Food scarcity is likewise known to have prevailed in an acute form in several other parts of India, particularly in the south, where extensive and severe cholera outbreaks also occurred

The increase in cholera incidence during 1941 was first recorded in the Ganges valley from where the disease spread to the Punjab Sind, and the North-West Frontier Provinces the following year showing the same epidemic prevalence.

responsible for a relatively high mortality both in the extreme north and south of India. Having reached the Punjab in 1924 for instance, cholera spread during the ensuing winter months farther north into Kashmir where it caused 11 504 deaths during 1925 i.e. practically as many as during the outbreak taking place in that region in 1919 when 11 516 deaths were recorded.

The 1925 outbreak in Kashmir reached its peak in April and continued until October of the same year the vast majority of cases occurring in the province of North Kashmir In the extreme south of India the disease reached its maximum in January 1925 during the north-east monsoon months. The incidence continued at a high level, and another maximum was reached in January 1926 The infection spread farther south to Ceylon

Epidemic conditions again began to develop early in 1927 in Bengal and Madras Presidency and the cholera incidence was on the increase from April onwards also in the United Provinces and in Bihar and Orissa. The spread westwards rapidly affected the Punjab While the epidemic showed a tendency to increase in these provinces, in the meantime cholera had spread to the Central Provinces, Hyderabad, and Bombay Presidency the first two of which were most severely affected in their western districts With the onset of a favourable season for cholera in Bengal and Madras, the incidence was again at a high level in November 1927 whereas in winter the incidence declined rapidly elsewhere in India By March 1928 cholera had reached its peak in Bengal and spread rapidly northwards into the Ganges valley through Bihar and the United Provinces to the Punjab the seasonal rise commencing earlier than usual In the Madras Presidency also an increase was observed which beginning in June 1928 affected mostly the south-eastern districts. By the end of the year cholera had spread westwards to Travancore State The worst affected areas, contributing nearly 90 / of the total deaths recorded in 1928 were the United Provinces, Bihar Orissa, Bengal, and the Madras Presidency

During 1930 cholera remained at an unusually high level in Bihar Orissa, and United Provinces although it showed a minor decrease in its endemic home in Bengal while in the Central Provinces and Bombay it prevailed in epidemic form and continued to persist through 1931 declining in 1932 to the lowest level recorded in the previous 60 years The very low figure of cholera mortality for 1933 is of particular interest in view of the fact that during this year a largely attended Kumbh festival religiously most important because it was held after an interval of twelve years, took place at Hardwar in the United Provinces As stated before as a rule such pilgrimages played a most distressing role in the dissemination of the infection.

The high cholera incidence in India during 1934-35 is attributable to the prevalence of epidemics developing once more in Bengal Bihar and Orissa the United and Central Provinces, as well as in Bombay and Madras.

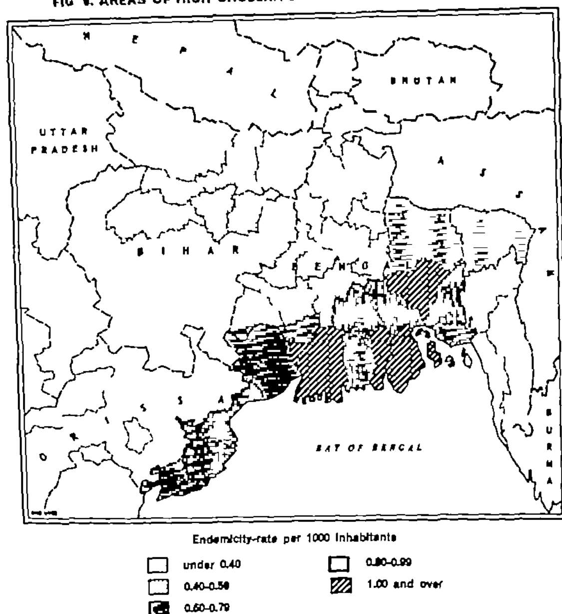


FIG 9. AREAS OF HIGH CHOLERA ENDEMICITY IN INDIA 1901-45

Although all the areas covered with dots in Fig. 1 (page 53) are to be regarded as favourable for the persistence of cholera in varying degrees Fig. 9 shows that the comparatively more markedly endemic zone is confined mainly to south and south-east Bengal lower Assam the coastal districts of Orissa and certain low lying regions in Bihar along the River Ganges

A more detailed examination of the cholera mortality figures for subdivisions of individual districts, the *thanas* enabled Swaroop (1951) to detect the existence of a major focus within south west Bengal at the confluence of three rivers, the Hooghly the Rupnarayan and the Damodar in a very densely populated and low lying tract of land situated about 40 miles south west of Calcutta (see Fig. 10) Lying on the opposite bank of the Hooghly River this focus is almost inaccessible from Calcutta during the

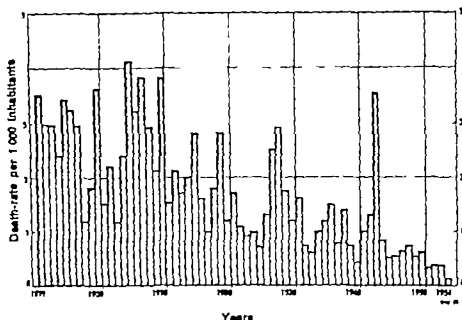
Out of a total of 459 930 cholera deaths during 1943 in India, Bengal alone contributed 47% Madras Presidency 25%, Bihar 11%, and Assam 3%. In Bengal, the epidemic was of such a severe nature that it did not even show its normal declin- during the monsoon months of July and August, but continued to rise steadily month after month to reach an acm in October

Even since 1948 cholera has continued to maintain a relatively high level *in different States—with the exception of the Punjab where the incidence* has shown a consistent decrease—and in some cases even to assume epi demic proportions, as for instance, in Bengal during 1948-50 Bihar during 1950 and 1952 53 Hyderabad Bombay and Madhya Pradesh (formerly Central Provinces) during 1953 Madras during 1948 and 1950 and Uttar Pradesh during 1946 1948 and 1952 53

The incidence had been high in 1952 in both Bihar and Uttar Pradesh, and in both these provinces it further increased in 1953 A feature of the most recent epidemic—in 1953—was that West Bengal was affected relatively mildly A westward spread of the infection took place early in 1953 to Madhya Pradesh, Hyderabad and Bombay where epidemic conditions prevailed throughout the year The epidemic reached its peak during the months of August and September A spread occurring further south to Madras was not of the same magnitude as that westwards to Bombay The disease crossed over to Ceylon in 1953 presumably from the port of Negapatam (now called Nagapattinam) whence sailing vessels had been arriving in Jaffna port, where illegal immigration into Ceylon had been discovered.

It will be clear from the foregoing account that up to the present day Bengal and the adjoining provinces continue to harbour the major endemic focus of cholera in the world. The focus of highest endemicity is, in fact situated in south Bengal in the delta of the Ganges and the Brahmaputra. A clear demarcation of the endemic zone is shown in Fig. 9 In order to compile this map the annual cholera mortality rates of individual districts in the whole of British India were studied over the period 1901 to 1945 For each district an average death-rate was worked out on the experience of those 15 years which recorded the lowest incidence out of the 45 years. This average cholera mortality rate called the " endemicity rate " permits a comparison of individual districts according to their level of endemicity In Fig. 9 only those districts which showed an endemicity rate of 0 40 per 1000 population or over have been shaded in varying degrees The four districts in which the endemicity rate exceeds 1 0 per 1000 are Howrah 24-Parganas, Bakarganj, and Dacca, the last two districts now belonging to East Pakistan Contiguous with these are the districts of Khulna, Tippera, Faridpur and Calcutta for which the endemicity rates were 0 99 0 99 0 97 and 0 92 respectively Two coastal districts of Orissa State, i e., Balasore and Cuttack, also show high endemicity rates of 0 91 and 0 81 respectively

FIG 11 ANNUAL CHOLERA DEATH RATE IN BENGAL, 1891 1954



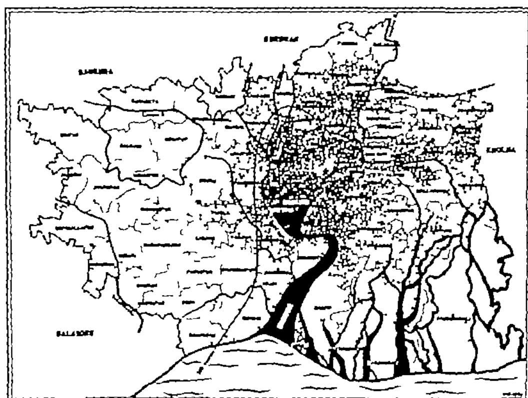
1944 the incidence has remained at a relatively much lower level as com pared with the experience in the previous quinquennia, the frequency of cholera during 1951 55 being only 10% of that in 1891 95

TABLE XIV CHOLERA MORTALITY RATES PER 1000 BY 5-YEAR PERIODS IN BENGAL ; 1891 1955

Period	Mean annual death-rate per 1000	Percentage of the 1891-95 rate
1891-95	3 00	100
1896-1900	2.54	85
1901-05	2.25	76
1906-10	3.16	105
1911 15	2 02	67
1916-20	1.68	56
1921 25	1 08	36
1926-30	1.92	64
1931 35	1 02	34
1936-40	0 94	31
1941-45	1 42	47
1946-50	0.59	20
1951-55	0 29	10

Provisional figures

FIG. 10. DEGREE OF CHOLERA ENDEMICITY IN BENGAL, 1934-48



Based on the average cholera mortality of the five years of lowest incidence.

major part of the year on account of floods and poor means of communication Nevertheless, the proximity of the focus to the city and the persistence of the disease in it at all times are undoubtedly factors responsible for the continued prevalence of cholera in Calcutta from year to year

In view of the low incidence of the disease recorded during the period 1945-54 it is not unlikely that the zones of cholera endemicity which, as noted before, have been demarcated through a study of the mortality figures prior to 1945 may have shrunk within recent years

Annual cholera mortality rates for Bengal from 1891 to 1954 are shown in Fig. 11 and the values of these rates for five-year periods are summarized in Table XIV

In the last column are set out what may be regarded as the index numbers of cholera mortality for each five year period the experience of the quinquennium 1891 95 being reckoned as 100 This index number and Fig. 11 serve to indicate a long-term tendency for the decrease of the cholera incidence from the beginning of the century—a trend that was upset during the 1941-43 Bengal famine years. As already stated, the 1943 epidemic occurred under rather unusual conditions marked by a virtual temporary breakdown of control measures during the famine period. Since

TABLE XV DECREASE IN CHOLERA MORTALITY RATE FROM 1901 10 TO 1934-43 BY DISTRICTS IN BENGAL

Districts	Average death-rate per 1000 population		Percentage decrease from 1901 10 to 1934-43
	1901 10	1934-43	
Burdwan	2 65	0.86	67.5
Birbhum	2.89	0.94	67.5
Bankura	1.65	0.77	53.3
Midnapore	3.26	0.83	74.5
Hooghly	2.27	0.81	64.3
Howrah	4 14	1 71	58.7
24 Parganas	4.04	1 70	57.9
Calcutta	2.91	1.00	75.6
Nadia	3.61	1.22	66.2
Murshidabad	2.65	1.08	59.2
Jessore	3.17	1.23	61.2
Khulna	3 09	1 46	52.8
Rajshahi	3.28	0.80	75.6
Dinajpur	0.89	0.25	71.9
Jalpaiguri	0.95	0.49	48.4
Darjeeling	0.32	0.04	87.5
Rangpur	1 48	1.02	31 1
Bogra	2 43	0.66	72.8
Pabna	2.92	1.90	34.9
Malda	2.53	0.50	80.2
Dacca	2.96	1.64	44.6
Mymensingh	3.10	1.30	58.1
Faridpur	2.76	2.08	24 6
Bakarganj	3.47	2.51	27 7
Chittagong	1.54	1.23	20.1
Noakhali	2.63	1 73	34.2
Tippera	2.06	1.53	25.7
Bengal	2.73	1.27	53.5

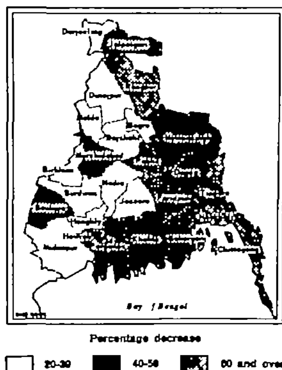
Calcutta heads the list with a rate 4 3 times that of Negapatam and 7 7 times that of the neighbouring port of Chittagong in East Pakistan

Fig. 13 shows the number of annual cholera deaths in Calcutta from 1841 to 1955 Throughout this period of over a century the city has not

A study of the rates of decrease in cholera mortality in individual districts of undivided Bengal reveals an interesting pattern In Table XV, cholera death rates are shown for each of the 27 districts of Bengal for the two 10-year periods 1901 10 and 1934-43 The last column gives the percentage decrease which has been observed in these two average rates.

The slowest decrease is shown in *Chittagong* (20%) followed by Farid pur (25%) Tippera (26%) and Bakarganj (28%) All these districts now belong to Pakistan and lie in a highly cholera-endemic area (Fig. 12) The other southern districts of Howrah 24-Parganas, and Khulna which also belong to the most endemic area in Bengal, record relatively slower rates of decrease. Table XV suggests that a relatively greater decrease has occurred in the less cholera-endemic zones.

FIG 12. PERCENTAGE DECREASE OF CHOLERA MORTALITY FROM 1901 10 TO 1934-43 IN BENGAL



Percentage decrease

20-30 40-50 60 and over

Cholera in Seaports

Annual figures of cholera cases reported in important ports during the period 1926-55 are shown in Table XVI It would seem that, with the exception of the port towns of India (including Pondicherry in former French India) and East Pakistan the incidence in seaports has become almost negligible. A study of the cholera-endemic level of various important seaports of south Asia gives the endemicity rates shown in Table XVII.

SEAPORTS OF ASIA FROM 1926 TO 1955

1940	1941	1942	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	1955
162						24	0	1	172	15	365	0	0	0	0
24						186	71	1	3	0	265	0	1	0	0
63						[illegible]4	4	2	3	2	34	10	5	0	1
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0						68	0	0	1s						
0						4 112	0	0	0						
911						514	6	0	0	0	0	0	0	0	0
0															
568						4 415	53	0	0						
0						260	0	0	0						
8						144	0	0	0						
0						107	0								
530						13	0	0	0	0	0	0	0	0	0
0	17	0	17	1	86	2	114	66	5	326	2	15	90	1	0
2 525	6 855	2 400	6 945	3 610	5 381	1 945	4 862	7 924	5 494	9 529	5 927	3 358	6 321	1 826	2 834
1	3	238	1 052	48	66	3	28	1 175	430	1 136	1 203	825	3 812	39	0
0	0	0	21	20	0	0	17	17	31	135	87	127	169	32	29
0	0	0	173	0	0	0	20	12	14	77	34	1	61	16	0
20	5	27	57	327	19	0	1	0	0	0	0	0	28	2	1
0						0	42	425	100	828	80	15	9		
4						8	34	36	75	187	65	39	5	82	64
63						0	0	2	0	0	0	0	0	0	0
0						503	718	0	8	0	0	0	0	0	0

In order to indicate the months in which the danger of a spread of the infection is potentially greatest, curves of the seasonal cholera incidence in ports where the disease has been more or less frequent in the recent past are shown in Fig. 14 These curves have been drawn on the assumption that 100 cases of cholera occurred in each of the five ports and show the percentage distribution of cholera cases in 13 four weekly periods of the year

TABLE XVI. CHOLERA CASES NOTIFIED IN VARIOUS

	1926	1927	1928	1929	1930	1931	1932	1933	1934	1935	1936	1937	1938	1939
Burma														
Bassein	151	55a	147a	206a	15	2	0	0	16	56	22	225	12	9
Moulmein	*0*	*0*	29a	68a	0	*1*	*0*	*6*	*0*	*47*	5	*0*	0	*0*
Rangoon	180	96	102	61	26	13	7	3	3	75	17	18	4	14
Ceylon														
Colombo	0	0	2	1	0	3	0	0	1	7	0	0	0	0
China														
Amoy	274	109	0	4	0	0	1 646	0	0	0	0	0	7	0
Canton	0	99	22	29	31	7	1 115	2	1	1	0	6 000	76a	0
Hong Kong	3	0	0	0	0	0	209	0	0	0	0	1 690	807	678
Port Arthur	2	0	0	0	0	0	11	0	0	0	0	0	0	0
Shanghai	1 440	3	38	3 258	115	325	3 978	0	3	0	0	3 408	11 638	435
Swatow	66	114	3	181a	8	18	689	2	0	6	0	216	625	0
Tientsin	0	2	0	1	0	0	103	103	0	0	0	0	268	33
Chinwangtao	0	8	0	13	0	0	15	1a	0	0	0	0	0	0
Macao	0	5a	0	0	0	0	100	0	0	0	0	615	1 236	668
India														
Bombay	6	133	39	11	57	130	11	17	7	8	2	0	0	0
Calcutta	1 962	3 021	3 716	4 316	2 965	2 163	[illegible]843	2 689	3 201	4 946	4 157	1 646	2 912	3 964
Madras	168	944	699	27	49	442	7	131	365	338	367	787	131	6
Negapatam	139a	64a	36a	25a	4a	10a	0	1a	2a	33	164	47	12	2
Tuticorin	1	52a	165	266	12	1	0	0	0	72	47	10	0	0
Vizagapatam	1	*0*	50a	*0*	0	*0*	0	15	*10*	1	*0*	*0*	1	*0*
French India														
Pondicherry	23	17	42	22	3	247	4	0	0	0	0	0	0	0
Pakistan														
Chittagong	11a	83a	62a	61a	61a	166	6	57	62	93	55	174	14	47
Karachi	0	0	0	11	0	0	0	0	0	0	0	0	0	0
Thailand														
Bangkok	2 193	250	454	465	68	17	3	3	1	175	580	1 861	0	1

a Deaths. *b* 10 months. *c* Suspected case. Figures not available.

been free from cholera for even a single year three to four thousand attacks being recorded annually on an average. During the famine year of 1943 Calcutta had 6945 cases but even this high figure was exceeded in two of the more recent years, i.e., 1948 and 1950, during which 7924 and 9529 cases respectively occurred. As shown by Table XVI the cholera situation continues to be serious at Calcutta and, to a lesser extent, also in Madras

SEAPORTS OF ASIA FROM 1926 TO 1955

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162						24	0	1	172	15	365	0	0	0	0
24						186	71	1	3	0	165	0	1	0	0
53						24	4	2	3	2	34	10	5	0	1
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0						68	0	0	1						
0						4 112	0	0	0						
911						514	6	0	0	0	0	0	0	0	0
0															
568						4 415	53	0	0						
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8						144	0	0	0						
0						107	0								
530						13	0	0	0	0	0	0	0	0	0
0	17	0	17	1	86	2	114	58	5	326	2	15	90	1	0
2 525	6 853	2 400	6 945	3 610	5 331	1 945	4 862	7 924	5 494	9 529	5 927	3 358	6 321	1 626	2 834
1	3	236	1 052	43	56	3	28	1 175	430	1 136	1 203	825	3 812	39	0
0	0	0	21	20	0	0	17	17	31	135	87	127	189	32	29
0	0	0	173	0	0	0	20	12	14	77	34	1	61	16	0
20	5	27	57	327	19	0	1	0	0	0	0	0	28	2	1
0						0	47	425	100	828	80	15	9		
4						8	34	36	75	187	65	39	5	82	64
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0						503	718	0	8	0	0	0	0	0	0

In order to indicate the months in which the danger of a spread of the infection is potentially greatest, curves of the seasonal cholera incidence in ports where the disease has been more or less frequent in the recent past, are shown in Fig 14 These curves have been drawn on the assumption that 100 cases of cholera occurred in each of the five ports and show the percentage distribution of cholera cases in 13 four weekly periods of the year

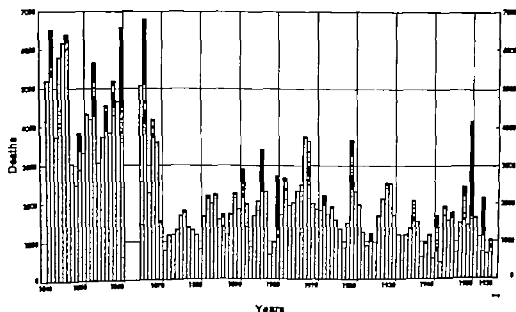
TABLE XVII. CHOLERA ENDEMICITY RATES IN VARIOUS SEAPORTS OF SOUTH ASIA, 1946-55

Port	Rate
Calcutta	83.8
Negapatam	19.4
Chittagong	10.9
Madras	0.7
Tuticorin	0.3
Rangoon	0.2
Bombay	0.04

Average annual cholera case-rates per 100 000 inhabitants for the five years of lowest prevalence during the period 1946-55.

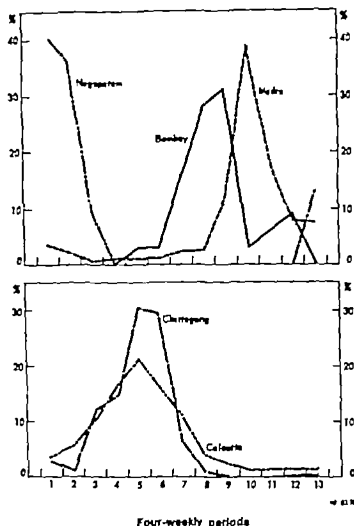
The seasonal variation in the incidence of the disease is similar in the ports of Calcutta and Chittagong, showing an increase during the months of May June and July The winter rise is almost negligible While the port of Chittagong has recorded several weeks free from cholera cases, it is significant to find that in Calcutta city even though the incidence declines following the monsoons and is low in the winter months, not a single month is known to have been free from cholera during the last half-century

FIG 13. ANNUAL CHOLERA DEATHS IN CALCUTTA, 1841 1955



Figures for 1861-64 are not available

FIG. 14. PERCENTAGE DISTRIBUTION OF ANNUAL CHOLERA CASES IN FOUR WEEKLY PERIODS, 1948-53



for which monthly figures of cholera cases have been examined Madras port shows its peak during the months of August and September, and Negapatam during the winter months at the time of the north-eastern mon soons Bombay port shows a peak during the south west monsoon period.

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identical view was taken in 1849 by Snow in a pamphlet entitled *On the mode of communication of cholera* As Snow stated in the second (1855) edition of this publication

"Diseases which are communicated from person to person are caused by some material which passes from the sick to the healthy and which has the property of increasing and multiplying in the systems of the persons it attacks."

Applying this concept to the pathogenesis of cholera, Snow came to the conclusion that

" the morbid matter of cholera having the property of reproducing its own kind, must necessarily have some sort of structure, most likely that of a cell. It is no objection to this view that the structure of the cholera poison cannot be recognised by the microscope, for the matter of smallpox and of chancre can only be recognised by their effects, and not by their physical properties "

A statement even surpassing in importance that of Snow was made in 1849 by Budd who as summarized by Macnamara, in a letter published on 5 September of that year in *The Times* expressed the opinion that the causative organisms of cholera were

"a distinct species of fungus which, being swallowed, becomes infinitely multiplied in the intestinal canal, and the action thus excited causes the flux of cholera, which with its consequences constitute the disease "

These organisms, Macnamara continued, Budd believed to be disseminated through society by their contact with food and principally by the drinking water of infected places and consequently he recommended as the most important means of preventing the progress of cholera that the poison which continues to be generated in the bodies of infected persons should be destroyed by mixing the discharges with some chemical compound such as sulfate of iron or chloride of lime known to be fatal to beings of the fungus tribe "*As water is the principal means of the dissemination of the* disease when it exists, too much care could not be exercised in procuring pure drinking water"

As stated by Sticker (1912) Pacini examining the intestines of cholera victims at the time of the 1854 outbreak in Florence claimed to have found a *microbio colerigeno* which had the property of destroying the epithelium and of entering into the deeper layers of the intestine, but not into the blood Since these bodies, in the warm dejecta showed a motility by far surpassing the velocity of Brownian movement, they represented no doubt, a *contagium animale*

Working at the same time as Pacini in St Thomas's Hospital, London, Hassall, as quoted by Sticker found

"myriads of vibriones in every drop of every sample of ricewater discharge of these vibriones many formed threads more or less twisted while others were aggregated into masses which under the microscope presented a dotted appearance"

These vibrios which were depicted by Hassall and which in Sticker's opinion represented true cholera vibrios, were absent from the blood or urine of the patients though abounding in their stools

As claimed by Sticker in 1866 true cholera vibrios were seen in the dejecta of patients by Leyden (see Wiewiorowski 1866) and in the vomits as well as in the stools of these sufferers by Bruberger (1867) Similarly according to a statement made by Virchow at the 1885 cholera conference in Berlin Klob in his work (1867) on the morbid anatomy of cholera, depicted and described intestinal organisms obviously identical with *V cholerae*

Feeling convinced of the validity of the views held by Snow and by Budd, Macnamara tried to obtain proof of the presence of cholera germs in the dejecta of the patients by orally infecting experimental animals. As was to be expected he was unsuccessful and had, moreover the misfortune of contracting the disease himself so that he had to go on leave to England preparatory to his retirement in 1876 However while continuing to work as a surgeon in London, he enlarged his knowledge of bacteriology by studying for some time under Koch in Berlin Anticipating the 1883 outbreak in Egypt he applied to the India Office for facilities in order to continue his cholera researches there It is tragic indeed that, as deplored by Rogers (1950) in a well documented article officialdom failed to comply with this request, thus bereaving one of the greatest authorities on cholera of the possibility of crowning his lifework by the detection of the germ causing this disease

However a French commission composed of Roux, Straus Nocard, and Thullier[1] as well as a German commission under Koch and Gaffky were sent in 1883 to Egypt. As stated by Chambers (1938) in a fascinating account of their work

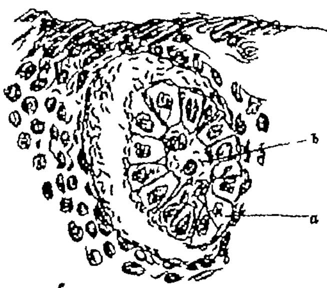
> "Discovery of the guilty microbe was the goal of each commission but they approached the problem from different angles. Koch, the pupil of Henle, who was in turn a pupil of Johannes Müller quite naturally approached the problem as a microscopic anatomist who had turned microbist He looked for the organisms that were invading the tissues about the intestinal lesions, culturing and isolating them. Roux, the pupil of Pasteur whose great work in animal diseases had been done by infecting laboratory animals, set out first to reproduce the disease in animals. It just so happened that in this particular disease Roux's method could not succeed because cholera is peculiarly a disease of man and animals do not have it. On the other hand, Koch's method in this particular disease was one of promise"

After the termination of the Egyptian epidemic, continuing his researches in India, Koch found that the peculiar bacilli he had suspected and isolated in Alexandria were invariably present in the dejecta of the cholera patients examined by him in Calcutta and in the intestines of victims of the disease,

[1] Sad to relate, Thullier a most promising young worker contracted cholera and succumbed.

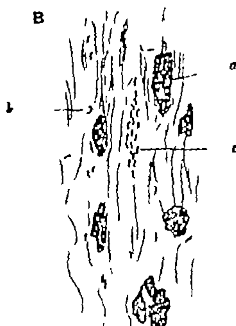
FIG 15. DRAWINGS OF FIRST SLIDE PREPARATIONS BY KOCH OF CHOLERA VIBRIOS

A



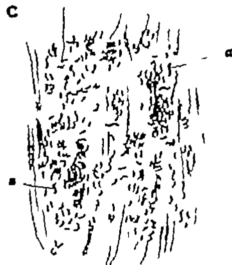
A. Cross-section of intestinal mucosa of cholera patient. A mucosal gland (a) has been cut obliquely within it (b) and between the epithelium and the basement membrane (c) are numerous comma bacilli. × 600.

B



B Cover-slip preparation of contents of intestine of cholera patient. The nucleus of the epithelial cell is destroyed (a) ; a comma bacillus is shown at (b) ; characteristic grouping of comma bacilli (c) × 600.

C



C. Cover-slip preparation of dejecta of cholera patient kept for two days on moist clothing, showing proliferation of comma bacilli, including some S-shaped bacilli (a) × 600.

D



D Cover-slip preparation from edge of drop of pure culture of comma bacilli on meat broth, showing long spiral forms (a) × 600.

Reproduced from Koch (1884a) by kind permission of the publishers

but were absent in any other morbid condition. The multiplication of these germs (first called "comma bacilli" on account of their curved aspect when examined under the microscope—see Fig 15) which regularly took place as the disease progressed and their disappearance in recovering patients also lent strong support to the contention that the organisms in question were responsible for the causation of cholera it still proved impossible, however, to confirm all Koch's postulates by using these organisms to induce the disease in experimental animals and isolating them again from the latter. Nevertheless Koch did not hesitate to report in February 1884 to the German Government that his labours during the cholera outbreaks in Egypt and at Calcutta had been fully successful (see Kleine 1934).

Though the validity of Koch's findings was soon widely acknowledged misgivings were expressed because, in contrast to his initial findings made under particularly favourable conditions, it was by no means invariably possible to demonstrate the comma like bacilli (or as they were soon called, the cholera vibrios) in individuals who were to all appearances affected with, or had succumbed to typical cholera. Worse still, findings such as those of Finkler & Prior (1884) during an 1884 cholera nostras outbreak at Bonn soon showed that in addition to the *Vibrio cholerae*, considered unique by Koch, vibrios more or less resembling it do abound and might—as claimed by several observers—be of etiological importance in the causation of gastro-intestinal affections. Indeed, one might claim that from 1884 onwards the study of cholera in the laboratory was to a large extent devoted to endeavouring to differentiate in a sufficiently accurate manner between the true cholera vibrios and cholera like vibrios. To show to what extent this goal has been reached is one of the main objects of this chapter and some of the later chapters.

Classification

According to Bergey's *Manual of determinative bacteriology* (1948) the classification of the cholera vibrio is as follows

Class	*Schizomycetes* Nägeli
Order	*Eubacteriales* Buchanan
Suborder	*Eubacteriineae* Breed, Murray and Hitchens
Family	*Pseudomonadaceae* Winslow et al.
Tribe	*Spirilleae* Kluyver and Van Niel
Genus	*Vibrio* Müller
Species	*Vibrio comma* (Schroeter) Winslow et al.
	(synonyms *Vibrio cholerae* Neisser
	Vibrio cholerae asiaticae Pfeiffer)

The common characteristics of the genus *Vibrio* (a term derived from the Latin verb *vibrare* to vibrate) are given in Bergey's manual thus

"Cells short, curved, single or united into spirals. Motile by means of a single polar flagellum, which is usually relatively short rarely two or three flagella in one tuft. They grow well and rapidly on the surface of standard culture media. Aerobic to anaerobic species. Mostly water forms, a few parasites."

Morphological Characteristics

Normal forms

A description of the appearance of *V cholerae* under the microscope must be qualified by the following statement "As long as the usual methods of examination exclusive of flagellar staining are implemented it is invariably impossible to distinguish between this organism and the allied members of the genus *Vibrio* because they all appear identical in this respect" In view of the marked pleomorphism displayed by the vibrios in general, and the cholera vibrio in particular, it is at the same time difficult to define the "typical" morphological appearance of the latter However as aptly described by Mackie (1929) recently isolated cholera vibrios, which had been grown at 37°C for 18-24 hours on carefully standardized agar media of an adequate alkalinity (e g with a pH of 8 0) are apt to appear

"as short, definitely curved cylindrical organisms with rounded or slightly tapering ends and measuring usually 1.5 to 2μ in length by 0.3 to 0 4μ in breadth"

It must be realized, however that even at best the microscopic preparations made from cholera material invariably show some evidence of pleomorphism Differences in the degree of curvature are bound to be noticeable under all circumstances because naturally those vibrios whose plane of curvature lies parallel to the level of the field of vision will appear more markedly bent than the organisms lying in other planes (Mackie, 1929) Moreover individual vibrios or even strains, may markedly vary in the degree of actual curvature so that instead of typically curved more or less straight forms may be present or even predominate. The length of the vibrios also varies from strain to strain so that either longer (and invariably slightly curved) or short (and markedly bent) forms are conspicuous or solely present. Even in recently isolated cultures occasionally quite short forms resembling cocco-bacilli are found (see for example, Seal, 1935) (Fig 16, 17 and 18)

Shorter or longer forms resembling in appearance the letter "S" are often seen. The occurrence of the former is due to the fact that the vibrios are not merely bent in one plane but also twisted, thus representing a part of a screw turn (Kolle & Prigge 1928), while the longer "S" forms are the result of the adherence of two vibrios, specially those which have not parted after transversal fission Such adherent vibrios may however not only appear in the "S" form, but may also form semicircles In old cultures, long spirals, due to the adherence of several vibrios may be conspicuous, but

FIG 16. 18-HOUR CULTURE OF *VIBRIO CHOLERAE* ON NUTRIENT AGAR

Reproduced by kind permission of J. Gallut, Institut Pasteur, Paris.



Cilia stained by Van Ermengem method. × 1000.

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such spiral filaments are absent in films made from recently isolated cultures. The occasional presence of such spirals in smears made from the dejecta of patients is apt to be due to contamination rather than to the adherence of true vibrios (Gruber 1887)

It is noteworthy that in smears made from the flakes of typical rice water like cholera stools the vibrios often show a characteristic arrangement lying with their long axes parallel " like fish in a stream " this being probably due to the viscosity of the mucus in which they had been embedded (see Sticker 1912)

While the vibrios seen in stool smears though apt to vary in their dimensions, usually show the typical curvature those in histological sections of the intestines as a rule have the appearance of short straight rods For this reason and also because spindle-shaped forms may be present, the vibrios in such sections may resemble glanders bacilli *(Malleomyces mallei)* a feature noted by Koch in his initial investigations

As far as the findings in smears from cultures are concerned it is important to note that the above-described " typical " appearances of some of the organisms seem indicative merely of a phase in their development Henrici (1925) who studied this problem with particular care distinguished between (*a*) an embryonic stage corresponding to the period of accelerated growth in which the vibrios were large and bacillary in form (*b*) an adult stage, characteristic of the period of a decreasing growth-rate during which typical vibrios were found and (*c*) a senescent stage during which the irregular forms described below became apparent or even predominant.

Earlier observations made in this connexion by Wherry (1905) showed that such morphological differences indicating successive growth phases may be demonstrable simultaneously in one and the same culture at the periphery of the growth, where active cell division took place the vibrios displayed a short and almost oval form, whereas the older forms in the centre of the growth tended to be more elongated and to undergo involution

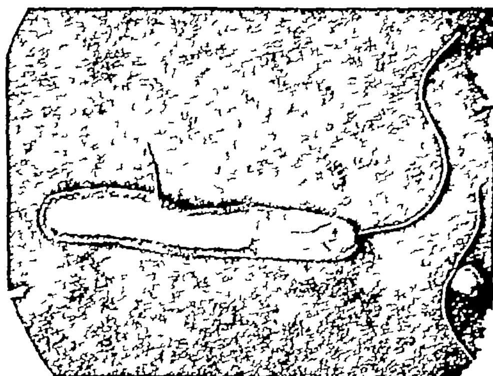
Envelopes and capsules

Observations suggestive of the formation of an envelope or capsule by the *V cholerae* seem to have been made under exceptional circumstances only [1] Using the flagellar stain recommended by Yokota (1924 1925) to study the opaque variant of this organism Balteanu (1926) found that some of the vibrios stained in this manner were surrounded by a thick layer of pink staining material When instead of using this stain the films were coloured for two minutes with carbol fuchsin, then treated for 10 to 20 seconds with 1 % hydrochloric acid and washed with water

[1] See, however the statement of Kolle & Prigge (1928), quoted later in the section dealing with the staining properties of *V cholerae* (page 105)

FIG 17 NORMAL FORM OF *VIBRIO CHOLERAE*, SHOWING CONDENSATION OF PROTOPLASM AND IMPLANTATION OF FLAGELLUM WITH COCCAL FORM AT RIGHT OF PHOTOGRAPH

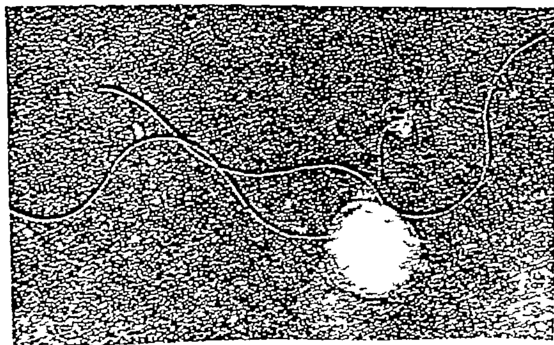
Reproduced by kind permission of J [illegible], Institut Pasteur Paris.



Electron microscope photograph × 50 000.

FIG. 18. GIANT COCCAL FORM OF *VIBRIO CHOLERAE* WITH FREE FLAGELLA

Reproduced by kind permission of J [illegible], Institut Pasteur Paris



Electron microscope photograph. × 50 000.

In conformity with a general rule the cholera vibrio is most actively motile at a temperature of 37°C Movements are slower at lower tempera tures and cease altogether at 16°C Riemsdijk (1929) found that the motility of cholera vibrios taken from the fluid beneath the surface pellicle of broth cultures was apt to be rather slight as compared to the active movements of the organisms collected from the pellicle It appeared therefore, that differences in oxygen supply exerted an important influence on the speed with which *V cholerae* progressed

As noted by some workers the organisms composing cultures which had undergone prolonged storage or variation leading to the formation of atypical colonies may show a loss of motility Nobechi (1923) who found two such immotile strains among the 88 stock cultures of *V cholerae* he examined established that this loss of motility was due to the absence of flagella. Balteanu (1926) who like Baerthlein (1912) before him described a variant of the cholera vibrio characterized by the formation of opaque colonies and as a rule also by complete loss of motility was of the opinion that the production of a slimy exudate by the vibrios in question explained " to some extent " the latter phenomenon It is important to note however that the organisms were found to possess no flagella.

Modes of multiplication

Elongation of the vibrios, followed by transversal fission is undoubtedly the usual and according to most workers even the only mode of multi plication of *V cholerae* However Braulke (1933) felt certain that longi tudinal division of the organisms may also occur The claim that reproduc tion may be affected as well by the formation of gonidia will be assessed in a later part of this chap er

Staining properties

It is certain that *V cholerae* which stains readily with all usual labora tory dyes is, as Mackie puts it, " definitely and uniform y Gram negative " The validity of this statement was corroborated by the investigations of Braulke who was able to establish that the occurrence of Gram positive cocci in vibrio cultures, instead of indicating the presence of the C forms postulated by Kuhn & Sternberg (1931) was actually the result of latent contaminations

As will be discussed in a later chapter several of the flagella staining methods have been found useful in cholera laboratory work. An interesting statement made in this connexion by Kolle & Prigge (1928) was that the cholera vibrios stained according to these methods appear much thicker than those stained in the usual manner because owing to the use of mordants for the former purpose the teguments as well as the bodies of the organisms become stained.

"the presence of a thick envelope stained pink surrounding the red vibrio was easily demonstrated. Sometimes a like covering enclosed two or several bacteria in a common matrix of mucus-like material. The opaque variant had evidently acquired the faculty of producing a slimy exudate simulating a capsule."

Further observations on this point have been recorded by Bruce White (1938) in a most valuable study on the rugose variant of *V cholerae* He stated that he had seen truly capsulated forms only in the case of a markedly atypical strain, whereas, generally, the organisms composing the rugose colonies appeared to be enclosed in a common zooglea of gelatinous or mucoid intercellular material. However ascribing the rugose condition of the cholera vibrios to an intensification of normal secretory processes, Bruce White maintained that no fundamental, but merely a gradual, difference existed between the truly capsulated forms and those embedded in a common matrix.

Flagella

Most cholera workers are in full agreement with Koch s original statement, confirmed by early observations in Egypt (see Kolle & Gotschlich, 1903) and ample subsequent work in India, that, in contrast with part of the cholera-like species *V cholerae* possesses only one polar flagellum. Statements made to the contrary by a few observers deserve no credence because they were never based upon results obtained with strains freshly isolated from patients but upon findings made in the case of growths which had undergone the vicissitudes of prolonged storage, in variants or in vibrios from carriers (see Seal, 1935) More important still, most, if not all, claims that the cholera vibrio may possess more than one flagellum were made before the now available reliable methods of serological identification could be implemented.

As summarized by Mackie (1929)

"the length of the flagellum is somewhat variable, measuring up to 4 or 5 times the length of the vibrio. Long flagella are frequent but short vibrios with short flagella may be seen. Kolle & Prigge (1927) have figured these two morphological types, namely short ovoid organisms with short flagella and longer forms with long flagella."

Motility

It is generally agreed that, if examined under the conditions specified below the cholera vibrios present in the dejecta of patients or in recently isolated cultures are invariably motile showing a "scintillating" movement, compared by Koch to the exceedingly rapid progress of a host of gnats and sometimes also exhibiting a "centrifuge" movement, consisting of a rotation on their short axes. Studying the rapidity of its locomotion, Sanarelli (1919) found that *V cholerae* was endowed with a speed three times greater than that of *Bacillus prodigiosus* five times that of *Salmonella typhosa*, ten times that of *Escherichia coli*, and twelve times that of *B megatherium*

"A variety of shapes may be observed, e.g. straight organisms, thicker and swollen individuals, spherical forms with faintly stained centres, spindle club- and pear-shaped organisms, individuals with irregular swellings, long spirals measuring up to 17μ, and cells which present a completely distorted structure"

In addition to these forms, Mackie referred also to the observation of spherical or triangular giant forms, branched filaments cladothrix like forms, and "budding" forms with roundish protuberances

In a recent article, describing the morphological changes taking place when cholera vibrios were kept in penicillin solutions (25-100 units/ml), Bruce White (1950) noted the appearance (*a*) of numerous globular forms at first 8-10μ in diameter and motile but enlarging upon prolonged incubation losing their motility as well as their staining properties, becoming vacuolated and apt to bulge in subsidiary masses from the periphery (*b*) under optimal conditions also of star fish like forms with tapering branches As noted by Bruce White,

"on staining the broader part of the branches is seen to consist of double or multiple chains of nucleus elements the finer branches are formed by single vibrios and from these the culture may regenerate, either in its original form, or if seeded on to fresh penicillin-agar in the spherical forms"

The question of what generally causes the above described morphological changes has been the subject of considerable debate The contention of a few authors that some of the abnormal forms, particularly the "budding" forms, might play a role in the perpetuation of *V cholerae* has been generally refuted In fact in the opinion of many observers these atypical forms were invariably the result of involution. Mackie, who was among those advocating the latter view, supported it by pointing out that these forms

"occur in cultures of some duration after growth has stopped and many of the individual organisms are dying and autolysing" and adding that "the various irregular forms described are such as might reasonably be expected to result from cell degeneration and particularly autolysis following death"

There can be no doubt that the above-described changes in morphological appearance are often the result of involution, the less so as it was sometimes possible to establish that the irregular forms which had developed under unsuitable conditions were incapable of multiplication At the same time however it would not be justifiable to claim that processes of involution play an exclusive role in this respect since evidence is available to show that cholera vibrios which had become morphologically atypical because they had been subsisting on exhausted media or in the presence of substances inimical to them were capable of reverting to type when grown once more under suitable conditions Particularly illuminating observations in this respect have been recorded by Braulke (1933) and recently by Paoletti (1952)

Nuclei granules and filtrable stage

Discussing the morphological characteristics of the cholera and allied vibrios, Peruzzi (1926) stated that these organisms may show differentiated chromatic bodies, presenting during division not only the appearances, but the behaviour of a nuclear apparatus. Korobkova (1931, 1936) studying the morphological aspect of cholera vibrios grown on a special potato-starch medium, also noted the occurrence of well-differentiated nuclei

In a recent publication Paoletti (1952) claimed to have demonstrated with the aid of the method of Robinow (1942, 1944) chromatic bodies in cholera vibrios similar to those described in other bacteria and considered by some workers to represent morphologically discrete nuclei

The presence of polar bodies in cholera vibrios has been noted by several observers, first apparently by Finkler & Prior (quoted by Sticker 1912) who reached the opinion that these *Polkörner* becoming liberated and sedimented after decay of the vibrios, could give rise to typical growths even after they had been subjected to prolonged exsiccation Similarly Hüppe (1885) postulated that the granules observable in cholera vibrios which had become transformed into filaments after exhaustion of the media, could assume the role of resistant " arthrospores " and thus become a link in the propagation of these vibrios However as stressed by Kolle & Prigge (1928) subsequent observations showed that cultures rich in such granules were no more resistant to exsiccation or other untoward influences (heat, disinfectants) than growths free from " arthrospores "

A similar view was also expressed by Braulke (1933) who found that the small spherical forms of the vibrios (*Kügelchen*) apt to become preponderant in old cultures, specially those constantly kept at 37°C, and suspected by some workers of acting as " gonidia " were actually unable to multiply

Braulke, like Bisceglie (1929) before him, was also unable to find evidence for the existence of a filtrable stage of the cholera vibrio which had been postulated by a few workers. In the opinion of Braulke, defects in the filter-candles they had used were responsible for the apparently positive results reported by these observers.

Morphological variation

Ample experience has shown that the cholera vibrios in old cultures as well as those grown in the presence of substances apt to prove inimical to their development, often display a morphological appearance markedly different from that found upon examination of fresh material.[1] As stated by Mackie (1929) under these circumstances

[1] The morphological changes resulting from dissociation of the *V. cholerae* will be described in a later section of this chapter

factory growth of *V cholerae* is apt to take place because they worked with different, or at least with differently prepared, nutrient media. Of great importance in this respect is also that, as shown by Kabelik & Freudmann (1923) the suitability of the media for the growth of the cholera vibrio depends upon an interrelation between the hydrogen ion concentration and the NaCl content, the optimum of the latter becoming lower as the former increases and *vice versa* As far as the plain media routinely used for cholera diagnosis, particularly peptone water, are concerned, however modern workers recommend an initial pH ranging from 8 0 to 9 0 or slightly higher (9 5 according to Matsuo 1924) Vedder & Van Dam (1932) assessing the value of Dieudonné agar (a selective medium described in a later chapter) for the cultivation of *V cholerae* found that no growth took place if the pH of the plates exceeded 9 6 This figure if fairly well in accord with the experience of Read et al (1939) when growing cholera vibrios in 1/100 peptone water with 1% NaCl according to which multiplication occurred up to a pH just in excess of 9 4 but with some reduction from 9 4 upwards. In the opinion of these workers

"a pH of 9.2 may therefore, be taken as the limit for satisfactory multiplication, a figure which is supported by the results of experience in isolating the vibrio from natural sources"

pH changes during cultivation That cultivation in the usual glucose-containing media leads to a marked lowering of the pH and that such a drop even takes place in the case of media into which no fermentable sugars have been incorporated is shown by the observations of Banerjee (1939) recorded below

Hours (or days) of growth	*Ordinary broth*	*0.2% glucose broth*
2	8.2	7 6
4	8.0	7 4
6	7 6	7.2
8	7 6	7.0
10	7 6	7.0
12	7 6	7.0
24	7 6	7.0
30 days	7 4	6 8

Remarks (*a*) The drop of the pH was uniform regardless of whether aerobic or anaerobic cultivation was resorted to (*b*) Banerjee found that, in contrast to the changes recorded above, no lowering of the pH took place when cultivation in Ramon s glucose medium (prepared from an HCl digest of hog s stomach and minced veal) was resorted to, the pH remaining at 8.2, apparently the original level.

Read et al. (1939) found that a pH of 6 0 marked the lower limit of the range within which multiplication of the cholera vibrio took place in peptone water Similarly Jennings & Linton (1944a) working with a medium which contained a casein digest besides inorganic salts and varying amounts of glucose found that most rapid growth of *V cholerae* took

The former worker replanting cholera vibrios previously grown on media containing lithium chloride on plain agar noted that at first mainly the abnormal "lithium forms" (small or large spheres, etc.) developed on the latter medium but that upon continued subcultivation these aberrant forms became rarer and were gradually replaced by typical vibrios.

More convincingly still, Paoletti, implanting material from old cholera cultures on new media and examining these subcultures at two-hourly intervals, was actually able to observe that the originally present round forms assumed first a quadrangular and then a sausage shape the latter forms becoming afterwards elongated, and finally converted into typical vibrios.

These and similar observations leave no room for doubt that the appearance of morphologically atypical forms of *V cholerae* besides being the result of involution or as will be shown later of dissociative processes, may be also indicative of a temporary adaptation of the organisms to unsuitable conditions (*Anpassungsformen* of the German writers)

Cultural Characteristics

Growth limits and requirements

Reaction of the media

When dealing with the reaction of the media suitable for the cultivation of *V cholerae*, attention must be devoted to (*a*) the initial pH of the fluids or solids used for this purpose and (*b*) the changes in the reaction of the media taking place in the course of cultivation. As will be shown later a high initial pH is of great importance for the primary isolation of the organisms, while in addition to this, a maintenance of not too low a hydrogen-ion concentration exerts a great influence, when cultivation of the vibrios in bulk is called for as for instance in the course of vaccine preparation.

Initial pH Though, as summarized by Pollitzer (1934b), "the *V cholerae* is not among the micro-organisms demanding elaborate preparations for their cultivation" it proves indiscriminating in this respect only as long as the media used for its growth possess a suitable pH. The presence of even a slight degree of acidity corresponding, according to Kitasato (quoted by Kolle & Prigge, 1928) to 0.07% HCl, is sufficient to impede the multiplication of the cholera vibrios. A markedly high alkalinity of the media, on the contrary is not only well tolerated by these and many other vibrios, but even proves most beneficial because it counteracts the growth of the contaminating bacteria often present in the specimens coming for examination.

It is not surprising to find that some variance exists in the statements made by different observers regarding the pH limits within which satis

Salt requirements

Beauverie (1916) cultivating cholera vibrios in broth with NaCl concentrations of 7 9 15 20 30 50 90 and 100 per 1000 respectively found growth to become visible within 24 hours in all concentrations up to but not above 50 per 1000 To judge from the formation of a particularly thick pellicle an NaCl concentration of 30 per 1000 was particularly favourable for the multiplication of the organisms. However Beauverie found that the cholera strains cultivated in broth with an NaCl content of 30-50 per 1000 while being at first favoured in their growth, aged quickly showing within a few days evidence of involution and loss of motility

As noted by Kabelik & Freudmann (1923) Sierakowski, in an article published in the *Przeglad epidemiologi* in 1922, had recorded that an NaCl concentration of 0.5% was optimal for the growth of *V cholerae* In Sierakowski s opinion, the discrepancy between his findings and those of Beauverie was due to the fact that, in contrast to Beauverie he had worked with solid media. However Kabelik & Freudmann making comparative tests with 2% peptone water ordinary broth, agar and gelatin with a varying NaCl content (0-6%) found that no fundamental differences existed between the solid and fluid media, as assumed by Sierakowski Like Beauverie they recommended the use of peptone water with an NaCl concentration of 3% for practical cholera laboratory work—a proposal also made more recently by Genevray & Bruneau (1938d)

Valuable investigations on the salt requirements of *Vibrio cholerae* were carried out by Read et al. (1939) who mainly used an artificial concentrated sea water solution for their tests, but also experimented with the components of this preparation (sodium chloride magnesium chloride magnesium sulfate, and potassium chloride) and with some other substances

Read and his colleagues established in the course of these investigations that

"In the absence of salt multiplication did not occur in any peptone concentration and in no case did survival reach 24 hours"

Multiplication of *V cholerae* was observed in the case of 1/50 000 peptone water at sea salt concentrations of 0 5% to 3%, in the case of 1/5000 peptone water at a salt concentration as low as 0 1% in that of 1/500 peptone water even at a salt concentration of 0.075%.

Testing individual salts (calcium chloride sodium nitrate and sodium sulfate as well as the above mentioned) Read et al. found that any one of the salts tested except magnesium sulphate can promote multiplication but none seems to do so in any specially low concentration and that the sea water solution mainly used for the experiments had a somewhat

place while the pH was falling from about 8 5 to 6 0 while lower as well as higher values were associated with inferior growth rates. Considering their experiences, Jennings & Linton suggested

" that while a high initial pH may be optimal in the sense that it gives the best conditions for an extended period of growth, the most desirable pH for rapid multiplication may lie in the region near neutrality Experiments showed that a pH of 10 was injurious to the vibrio and usually prevented growth altogether Invariably growth stopped when a pH of 5.5 was attained, whether at the end of a long vigorous growth starting at high pH or at the end of a shorter period when growth was initiated at a lower pH level."

Various procedures have been used to counteract the lowering of the pH which takes place in the course of the cultivations of *V cholerae* Some workers resorted to the periodic addition of alkalis to the cultures, Hirsch (1928) for instance, using calcium carbonate for this purpose Veeraraghavan (1949) sodium bicarbonate with the aid of which it was possible to maintain the pH of the special medium he employed (see later) at a pH level of 8 0 with a marked growth increase Various buffer substances have been incorporated in the media by other workers. Jennings & Linton (1944b) who as will be described below worked with a simple medium containing a casein digest besides glucose used " aeration " with a mixture of air and CO_2 to maintain the pH of their cholera cultures at a satisfactory level. The efficacy of this procedure was confirmed by Ranta & McLeod (1950)

Gallut (1947a) admitted that vigorous bubbling of air through the media promoted the growth of *V cholerae* by maintaining the oxidation reduction potential of the organisms at a constant level even in the presence of glucose, which in this case was oxidized instead of being fermented. However in a subsequent paper (1947b) he adduced doubts whether cultivation under enforced aeration was advantageous for the purposes of cholera vaccine manufacture because the vibrios grown with the aid of this procedure showed an atypical morphology (prevalence of filamentous forms and early appearance of global forms) as well as diminution of their nitrogen content.

Recently studying again the growth and survival of the cholera vibrio in relation to pH Sarkar & Tribedi (1954) drew attention to the fact that

" Acid production by the vibrio which determines the pH of the culture fluid depends (apart from other factors such as presence of fermentable sugar or other constituents, amount of growth, etc.) on the available surface area of the medium and therefore oxygenation. pH of 24 hours broth culture of *V cholerae* in 30 cc. nutrient broth of pH 7 6 in a test tube (1½ inches diameter) became 6 1 whereas pH of the same in Roux flasks (5½ inches diameter) became 8.2. *Growth was also much heavier in the latter* "

Further reference to the pH adjustment of the media used for the cultivation of *V cholerae* will be made in chapter 7 where the practical aspects of cholera laboratory work will be discussed

imposed Banerjee found that a restricted growth of *V cholerae* took place in glucose free broth even when the access of air to the culture medium had been prevented The evidence Banerjee obtained in this respect may thus be summarized

Hours of growth	*Growth of V cholerae in millions*	
	aerobic	*anaerobic*
3	50	12
6	500	40
9	2 200	100
12	4 500	240

In view of the evidence adduced above, the cholera vibrio must be considered a facultatively anaerobic rather than an obligatorily aerobic, organism

Temperature requirements

In contrast to certain other vibrio species (e.g. the one found to be the causative organism of a fish epizootic by David (1927) which grew best at 8°-20°C) multiplication of *V cholerae* is most abundant within a tempera ture range of 30°-40°C with an optimum at about 37°C. Growth at 22°-25°C, i.e at temperatures used mainly for the incubation of gelatin plates, is still fairly satisfactory

As claimed by Koch (1884) the cholera vibrios are unable to multiply at temperatures below 16°C. However as summarized by Kasansky (1895) it was soon shown by some other workers that a slow growth of *V cholerae* may still take place at temperatures ranging from 8°C to 15°C In Kasansky s own experience a multiplication of the organisms still occurred at 10°C to 12°C Moreover as will be seen in the concluding part of this chapter the cholera vibrios show a most remarkable resistance to low tem peratures, which are apt to prolong the life span of the organisms even though no longer permitting their multiplication.

Nutritional requirements

The efforts made by a number of workers to determine basic nutritional requirements of the *V cholerae* by cultivating it in simple chemically defined media, may be said to fall into two categories (*a*) attempts to grow this organism in media containing only ammonium salts but no amino-acids (*b*) use of media in which amino-acids were the essential constituents These two classes of investigations will now be dealt with seriatim.

(*a*) *Ammonium salts* Anderson, in *An introduction to bacteriological chemistry* (1946) stated that, like certain other bacteria, the vibrios comprise two types of strains (1) " exacting " strains, which require amino-acids for their growth, and (2) " non-exacting " strains, capable of growing on ammonium salts as well as on amino-acids. Anderson added that " the exacting strains are usually pathogenic "

higher capacity for promoting multiplication than the individual salts tested.

Magnesium sulfate besides being shown to be incapable of promoting multiplication or survival of *V cholerae* was also found to be rapidly lethal to the vibrios in the higher concentrations tested. Sodium sulfide (Na_2S), on the other hand if added to 1/50 000 peptone water in a strength of 0 0003%, secured multiplication of the cholera vibrio at a sea-salt concentration of 0.05 %, while 0 00003% Na_2S was sufficient to secure growth of the vibrios at a sea salt concentration of 0 1%.

Oxygen requirements

The dependency of the cholera vibrio on the presence of oxygen attracted the attention of Koch (1884) who placed pieces of mica on gelatin plates inoculated with *V cholerae* and noted that the practical absence of growth under these platelets stood in marked contrast with the abundant development of the organisms round them The early observers were also impressed by the fact that, when cultivated in fluid media, the vibrios grew most abundantly at the surface of these and were led to believe that an avidity of the organisms for oxygen fully explained why typically a pellicle formed on the surface of these cultures. Hesse (1893) carrying out gas analytic studies, reached the conclusion that the *V cholerae* was unable to grow in the total absence of oxygen.

However carrying out studies on the oxygen requirements of the cholera vibrio Hirsch (1926a) found that this organism was able to grow anaerobically as well as aerobically in a simple chemically-defined medium provided that a fermentable sugar (glucose) had been added to the latter These observations, he maintained, went a long way to explain how the vibrios could multiply in the intestine under what amounted practically to anaerobic conditions.

Carrying out further investigations on the metabolism of the cholera vibrio under aerobic and anaerobic conditions, Hirsch (1928) reached the following conclusions

"1 The aerophilic behaviour of the *V cholerae* in carbohydrate-free amino-acid solutions or in peptone solutions is conditioned in an obligatory manner by the chemical composition of the substrate and cannot be considered a specific property of the organism.

"2. The *V cholerae* is capable of multiplication under strictly anaerobic conditions provided that carbohydrates are available as an anoxybiotic source of energy" [Trans.]

Working with broth media, Banerjee (1939) fully confirmed the contention of Hirsch that the *V cholerae* was capable of growing luxuriantly under anaerobic as well as under aerobic conditions in the presence of glucose Moreover comparing the growth of this organism in ordinary broth tubes and in tubes in which a layer of vaseline oil had been super

the sole source of nitrogen also contained glucose The other 79 strains (including 71 cholera strains) required in addition purines for their growth hypoxanthine being the simplest of these substances which proved adequate It was further established that the purine requiring strains were capable of growing on inorganic media if human rabbit or goat serum was incorporated The sera of rats mice guinea pigs or horses failed to support the growth of these strains

(b) *Amino-acids* As summarized by Hirsch (1926b) Uschinsky (1893) was the first to cultivate cholera vibrios in a chemically defined fluid medium which contained NaCl, calcium chloride magnesium sulfate and dipotassium hydrogen phosphate as well as glycerol ammonium lactate, and sodium aspartate Fraenkel (1894) established, however that out of these substances only three, namely NaCl dipotassium hydrogen phosphate and a salt of aspartic acid were indispensable for the growth of *V cholerae*

Hirsch (1926b) making further careful studies of this subject, found that this organism was able to use *l* aspartic acid as the sole nitrogen and carbon source for its metabolic and energy requirements and that the decomposition of this acid was the result of an oxidative process, the end products of which were ammonia acetic acid and carbonic acid.

Further exhaustive investigations in this field were recently undertaken by Ranta & McLeod (1950) who tested 20 different amino-acids by adding them singly or in combination, to a basic medium containing 5 g of sodium chloride, 0 75 g of dipotassium hydrogen phosphate, and 0 1 g of magnesium sulfate in a litre of distilled water While confirming that asparagine gave relatively the best results, if used singly combinations of two or several amino-acids proved more satisfactory Ranta & McLeod recommended ultimately a medium containing 0 067% tyrosine, 0 051% glycine 0 042% asparagine, and 1% glucose

Agarwala, Krishna Murti & Shrivastava (1953) established in the course of a recent study on the oxidative metabolism of cholera and allied vibrios that cysteine threonine and asparagine were oxidized as at fast a rate as glucose lactate and pyruvate Basic amino-acids showed very little oxygen consumption.

Accessory growth factors

As will be gathered from the statements made above accessory growth factors (" bacterial vitamins ") are not indispensable for the cultivation of the *V cholerae* However Veeraraghavan (1949), using a chemically defined medium which contained, besides different salts ammonium sulfate and *l*-cystine noted that addition of Marmite (an autolyzed yeast product containing anti neuritic vitamin) had a growth promoting effect. Substitution of this substance by thiamine hydrochloride nicotinic acid pyridoxine hydrochloride calcium panthothenate riboflavin, biotin, and

The evidence available in this respect regarding the *V cholerae* may thus be summarized Kisch (1919) in contrast with some other early observers was able to cultivate this organism on a basic agar medium to which 0 19% ammonium sulfate or 0 262% ammonium tartrate had been added while he obtained no growth on the basic agar alone He postulated, therefore that the cholera vibrio was facultatively capable of growing in the presence of ammonium salts instead of organic nitrogen compounds.

Linton & Jennings (1944) and Jennings & Linton (1944a) who cultivated cholera vibrios in media containing, besides ammonium sulfate, other organic salts and glucose, as well as either peptone or a casein digest, came on the contrary to the conclusion that ammonium sulfate acted as a buffer rather than as an essential nutrient. As stated by Linton & Jennings growth took place if this chemical had been omitted from the media, while, on the other hand *V cholerae* failed to grow in the presence of ammonium sulfate but the absence of either peptone or casein digest solution. However in a later paper Jennings & Linton (1944b) admitted that they had found

" that very good growth could be obtained occasionally when no nitrogeneous matter other than supplied by the inoculum was incorporated in the medium. The irregularity of results, however prompted us to include the additional casein digest as a routine procedure, since the material could be completely removed by dialysis when desired."

It is of great interest to note that recently Saxena et al. (1953) recorded constant success when cultivating 14 cholera strains as well as one El Tor strain one strain of water vibrios, and one " rough " (? cholera) strain in a medium of pH 8 0 made up according to the following formula

Ammonium phosphate ($NH_4H_2PO_4$)	0 1 g
Glucose	0.1 g
Sodium chloride (NaCl)	0.5 g
Magnesium sulfate ($MgSO_4.7H_2O$)	0.02 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0 1 g
Distilled water to make	100 ml

Note Ammonium phosphate was found to give a better yield after 24-hour incubation at 37°C than ammonium sulfate.

While the observations of Saxena et al. prove that cholera vibrios can utilize ammonium salts as their sole nitrogen source it is important to note that growth in the above mentioned medium took place only when glucose was present and when large inocula (" at or above 100 million of organisms per cc. of medium ") were used.

The observations of Saxena and colleagues were confirmed by Bhaskaran & Rowley (1956) in that these two workers found that out of a total of 158 vibrio strains (including 148 cholera strains) half were able to grow on a simple inorganic medium, which, besides containing ammonium ions as

Growth in plain media

While it is proposed to deal with the special media recommended for the rapid isolation or bulk cultivation of *V cholerae* in a later chapter, it seems indicated at the present juncture to describe the cultural appear ances of this organism on plain media.

Peptone water

The great value of the method of cultivation or one should rather say, of enrichment in alkaline peptone water for the laboratory diagnosis of cholera is due to two fundamental properties of the *V cholerae* namely, (*a*) that this organism rapidly grows in the medium thus out-distancing the contaminating bacteria usually present in the specimens coming for exami nation, and (*b*) that the cholera vibrios have a most marked tendency to grow on and near the surface of fluid media.[1] Hence if one puts a particle of cholera-suspect stools, preferably a mucus flake or a minute quantity of other materials to be examined into a tube containing 1% 2% alkaline peptone water with an NaCl concentration of 0 5% (or more up to 3%) incubates the tube for a few hours at 37°C, then takes one loopful of material from the surface of the culture, transfers this into a fresh peptone water tube and after further incubation for some hours, takes a loopful from the surface of the subculture and uses this material to inoculate plates containing a suitable solid medium one may expect, in positive cases, to find on the plates the cholera vibrios in pure culture or at least in a sufficient degree of purity to permit the immediate application of confirmatory tests.

Considerable divergence of opinion exists as to the length of time during which the initial peptone water cultures and the subcultures made from these should be kept in the incubator before material for cultivation on solid media is taken from them As far as the original publications of the pioneers in this field could be consulted early workers such as Bujwid (1888) while taking full advantage of serial transfer incubated their peptone water cultures and subcultures for 24 hours before making use of them. Koch, in an 1893 article on the state of cholera diagnosis postulated in this connexion that

"the best time for examining the peptone solution is 6-12 hours after inoculation, [but] sometimes one must wait longer As a matter of fact it is necessary to examine a specimen from time to time, in order to establish the maximal development of the cholera bacteria. Later these are overgrown and replaced by other bacteria even in the upper layers of the fluid and it may happen that they cannot be demonstrated by too late an exami-nation" [Trans.]

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[1] Schottelius (1885), who seems to have been the first to have noted this tendency of *V cholerae* for surface growth, took diagnostic advantage of it by mixing 100-200 ml of suspect dejecta with 200-500 ml of alkaline broth or 10 times diluted meat-peptone-gelatin. The mixture was put into a beaker or even into a beer glass and left standing on or near a stove at a temperature of less than 40°C for 10-12 hours. Small quantities where then taken with the aid of a glass rod from the surface of the growth and used for examina-tion in hanging-drop or fixed and stained preparations.

yeast nucleic acid, singly or in combination did not give equally good results. It has to be noted however that, as stated by Anderson (1946), *V cholerae is capable of synthesizing some of these substances such as nicotinic* acid and biotin in the course of cultivation in chemically defined media which originally contained none of these compounds

Role of glucose

As will be gathered from some of the observations recorded above, the incorporation of glucose into the media used for the cultivation of *V cholerae* was apt not only considerably to promote the multiplication of the organisms but even to render their growth possible under conditions otherwise unsuitable for their propagation The disadvantage that addition of this *sugar to the media is* apt *to lead in the course of cultivation to* a particularly rapid and marked drop of their initial pH is more than compensated by the impetus the presence of glucose gives to the growth of the cholera vibrio

Hirsch (1928) devoting particular attention to the role of glucose in a valuable study on the metabolism of the *V cholerae* during aerobic and anaerobic cultivation established, in addition to the findings already recorded (see section on oxygen requirements page 112) that, under aerobic as well as under anaerobic conditions, the cholera vibrios by far preferred carbohydrates to amino-acids as sources of energy Accordingly the amino-acids (or the peptone) in media which also contained glucose were *mainly important* as *nitrogen sources As already referred to* Hirsch considered the aerobic growth of *V cholerae* in glucose-free media as a conditioned process forced upon the organisms by the limitation of the nutritive substances at their disposal. He felt convinced that not this process but growth under anaerobic conditions in the presence of glucose represented the natural mode of existence of the cholera vibrios in the infected intestine The present writer for one is in full agreement with this contention

Describing their experiences with the casein digest they originally used without aeration Jennings & Linton (1944a) concluded

" that glucose serves as an important source of the energy needed for reproduction (of the *V cholerae*), and *that it is utilized in a manner which results in the accumulation of* acid in the medium. The organisms are capable of using about 3 grams of glucose per liter before growth is stopped.

However when growing cholera vibrios in their modified aerated medium, Jennings & Linton (1944b) found that the new cultures could utilize as much as 10 g of glucose per litre, lesser concentrations of the sugar resulting in smaller final crops Addition of more than 10 g glucose *per litre did* not lead to an appreciable *increase of the yield.*

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Besides producing a general turbidity in broth or peptone water growth of *V. cholerae* may under suitable conditions also lead even more rapidly to the formation of a pellicle on the surface of these fluid media which as described by Mackie (1929) is at first semi-transparent and fragile but gradually becomes thicker and more coherent and may after an incubation of several days finally sink down in the media

It was often held that this formation of a surface pellicle was " a phenomenon of surface growth by an organism greedy of oxygen " (Iyengar 1920) but as shown by the systematic studies of this worker with broth media prepared with different ingredients and possessing varying degrees of alkalinity or acidity the presence or absence of a pellicle depended on the one hand of the reaction of the media used and on the other on the degree of nutrition they afforded to the organisms Thus in mutton broth prepared by tryptic digestion with an alkalinity above the neutral point of Eyre's scale (corresponding to a pH of about 8.2) pellicle formation as well as growth of the cholera vibrio were marked while the slight growth of the organisms in acid mutton broth was not accompanied by formation of a pellicle The latter remained absent in all broth cultures prepared with beef extract and Witte peptone in which weak growth of the cholera vibrios took place when the reaction was alkaline but no growth occurred when the media were acid

Mackie besides shortly summarizing the above findings of Iyengar and noting that in the experience of Beauverie (1916) an NaCl concentration of 3% promoted the surface growth of the cholera vibrio also drew attention to the observation of Wherry (1905) that " the property of pellicle formation could be established by serial transfers from the surface growth i.e., by a process of artificial selection " Presumably this phenomenon was the result of cultural variation which as will be discussed later may lead to marked changes in the growth appearances of *V. cholerae* in fluid as well as on solid media

Agar

The colonies of cholera and allied vibrios cultivated on agar plates from fresh materials such as the dejecta of patients or contaminated water typically show a rather characteristic appearance which permits their macroscopic distinction from the often simultaneously developing colonies of contaminating bacteria such as *E. coli* The vibrio colonies, after 18-24 hours incubation at 37°C appear as regularly circular pale discs, 1 2 mm in diameter which show a peculiar bluish lustre (*opalescierendes Irisieren* according to Kolle & Prigge 1928) when viewed in transparent light If incubation is continued the colonies attain a larger diameter (5-7 mm) and may eventually assume a yellowish brown coloration

Though shorter periods were considered permissible by some subsequent workers, e g. three hours by Dunbar (1896) and four hours by Babes (1914) Kolle & Prigge (1928) recommended incubation of the peptone water cultures and subcultures for six hours, while Mackie (1929) advocated a *period of six to eight hours However in the experience of the present writer* it is permissible, during epidemics particularly to use an incubation period of three hours only if one takes the precaution of continuing incubation of the peptone water cultures and subcultures so that material may be taken from them once more, should exceptionally the need arise Enriched surface water samples examined with the aid of such shortened incubation yielded analogously satisfactory growth of the cholera like vibrios abound ing in the rivers ponds, etc., of China.

That the growth of *V cholerae* in peptone water is rapid indeed has been demonstrated with the aid of biochemical methods by Dunham (1887) and Wakamiya (1940) The former found that weakly alkaline 1% peptone water with an NaCl concentration of 0 5% gave a definite, though slight, cholera red *reaction, if incubated for 4½ hours after inoculation* with cholera vibrios Wakamiya, making comparative test cultivations of *V cholerae* and *E coli* in peptone water into which an indicator system had been incorporated found colour changes in the case of the former organism after 1 hour s incubation, and in the case of *E coli* after about 3 hours of growth.

The sensitivity of tests with peptone water is well shown by observations *quoted by Mackie from a report issued by the British Medical Research* Council in 1920 according to which *V cholerae* could be demonstrated by enrichment in this medium when only four to eight vibrios were present in 25 ml of a dense faecal emulsion.

Since the growth appearances of the cholera vibrio in peptone water are identical with those in nutrient broth, they will be described when dealing with the latter medium Further reference to the practical use of peptone water *for the laboratory diagnosis* of cholera will be *made in a later chapter*

Nutrient broth

As has been noted above, multiplication of the *V cholerae* in suitable fluid media takes place with such rapidity that it is possible to start sub-cultivation from them as early as three hours after inoculation. This is all the more remarkable because, as confirmed by the experiences of the present *writer as a rule during the first few hours of* incubation no gross evidence of growth becomes manifest in the broth or peptone water tubes or flasks inoculated with cholera materials It is but *gradually usually* after a growth of 12 24 hours at 37°C, that a uniform turbidity develops in such cultures If one takes care not to shake the tubes, one may some times see that at first this turbidity is restricted to the uppermost stratum of the fluids where the most active growth of the vibrios takes place.

Coagulated blood serum

Incubation at 37°C leads to a rapid growth of *V. cholerae* on coagulated blood serum which is initially similar to that on agar. An important difference, however, is that in the case of the former medium liquefaction commences after 24 hours and gradually becomes complete. As aptly stated by Mackie (1929) the property of liquefying coagulated serum which like that of gelatin liquefaction is due to the proteolytic action of *V. cholerae* also shows the same range of variability recently isolated strains differing in the rapidity of liquefaction while old often subcultivated strains may more or less fail to produce this phenomenon.

Potato slopes

On potato slopes alkalinized by steaming in 0.7% sodium carbonate solution fairly abundant growth of *V. cholerae* is usually obtained after incubation at 37°C. In the course of this chromogenesis becomes frequently marked so that finally a yellow, greyish yellow, yellowish brown or pink coloration results.

As has been noted above a yellowish brown coloration also becomes manifest when agar cultures of *V. cholerae* are kept at 37°C for several days.[1] Bearing these observations and the experiences with potato slopes in mind Mackie was disinclined to consider pigment production as a sign characteristic enough to distinguish between the cholera vibrios and certain other vibrios exhibiting marked pigment formation as had been proposed by Chalmers & Waterfield (1916). As Mackie pointed out with great reason

"The property of chromogenesis seems a general one in the genus (Vibrio) though more pronounced in certain species according to the medium in which the organism is growing."

Milk

While it is generally agreed that milk is a suitable substrate for the cultivation of *V. cholerae* markedly divergent opinions have been expressed regarding the growth appearances produced by the organisms in this medium, a few writers even maintaining that in contrast to their usual behaviour the cholera vibrios do not produce an acid reaction in milk media. It has to be noted, however, that the evidence to the contrary brought forward by early workers, such as Kitasato (1889b), Schoffer (1895), Wherry (1905) and Kendall et al. (1914) has been fully confirmed through more recent observations by Pollitzer (1935) and Genevray & Bruneau (1938c). The former worker, carrying out a systematic study at Shanghai, found that

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[1] Genevray & Bruneau (1938c), studying about 500 cholera strains isolated during the 1937-38 epidemic in Tonkin, even found that when young agar growths were taken up in large quantity on a platinum loop a salmon-pink tint became noticeable.

As described by Mackie (1929)

" *stroke growth on an agar slope* after 24 hours incubation consists of an abundant, moist, semi-transparent confluent layer which is greyish-white in colour on continued incubation it becomes more raised and assumes a greyish-yellow tint which deepens after about 10 days to a brown colour "

Gelatin plates

As summarized by Pollitzer (1934b)

" The growth of cholera vibrios on gelatin plates is even more characteristic than that on agar Though it is necessary to incubate gelatin dishes at the comparatively low temperature of 22°C., one may after 24 hours macroscopically discern colonies represented by very small clear dots Seen under low power of the microscope they show a peculiarly granulated surface, as if strewn with glass particles (R. Koch). Macroscopically their transparency is in strict contrast to the opaqueness of the bacterial colonies one is apt to encounter when cultivating from faeces. On observing vibrio colonies of recent origin and typical behaviour one notes after about 48 hours a commencing liquefaction of the medium, the colonies appearing to sink into the medium and finally to lie in a small cup or funnel. The process of liquefaction continues, the whole medium becoming dissolved after about 10 days."

While at first glance these peculiar growth phenomena appear to be of great differential diagnostic importance, it has to be realized that they are characteristic of many of the cholera like as well as of the true cholera vibrios Moreover while even freshly isolated cholera strains may show some variation in the rapidity of gelatin liquefaction, the property of liquefying these media may be more or less completely lost by old, often subcultivated, strains A still greater drawback from the practical point of view is that at the temperatures usually prevailing during cholera outbreaks it is rather difficult to work with gelatin media. It is therefore not surprising to find that their use, though much relied upon during the years immediately following the discovery of *V cholerae* has now been given up in favour of other methods of cultivation

Gelatin stab culture

In gelatin stab cultures growth occurs along the whole track of the needle but is most marked near the surface where—as a result of liquefaction and evaporation—an air bubble forms within the gelatin. In the past, great stress has been laid upon the diagnostic importance of a typical evolution of this phenomenon. It has now been realized that an important influence is exerted by the composition of the media as well as by the quantity of the inocula used, and that, moreover cholera like vibrios may show a behaviour in gelatin stab cultures indistinguishable from that of true cholera vibrios (Pollitzer 1934b)

Kind of strain		Number of strains	Behaviour in milk
Strains from	India Indo-China	9 18	Grew abundantly producing acidity but caused with the exception of one Indian strain no coagulation in milk
Strains from minor outbreaks in Baghdad and Basorah		12	More or less complete coagulation within 2 24 days
El Tor strains [1]		27	Massive coagulation mostly within 24-48 hours in a minority within 3 15 days

Mustapha suggested that since milk coagulation was produced by the El Tor strains, which were as a rule non pathogenic and less rapidly also by strains isolated in the Middle East during minor cholera outbreaks but practically never in India or Indochina tests with milk media might be a means of distinguishing between cholera strains endowed with marked and low cholerigenic powers respectively However the validity of this assumption is disproved by the observations made in the case of more than 500 Indochina strains by Genevray & Bruneau (1938c) who reported on their experiences with milk media thus

" All the strains studied change the colour of litmus milk to pink within 24-48 hours and coagulate it. This coagulation commences with the formation of a coagulum cap on the surface of the medium, which often appears within 24-36 hours. The coagulum then slowly grows, reaching a thickness of 2 3 cm within 8 days. If left in the incubator the whole of the milk becomes coagulated and the cap more or less shrinks, sometimes undergoing a slight digestion. It has then an aspect similar to that of the soft part of bread." [Trans.]

The question of whether the coagulation of milk by *V cholerae* was the result of a rapid formation of acid alone or was partly or even wholly due to the slower action of a rennet like ferment which according to Fokker (1892) was produced by the cholera vibrios has been systematically studied by Schoffer (1895) Making parallel tests with milk samples to which lactic acid alone had been added at varying concentrations and with similar samples in which cholera vibrios were cultivated he established that the organisms were apt to cause curdling of milk at much lower degrees of acidity than one would have expected from the tests with lactic acid The action of ferments was, therefore apparently of paramount, if not of sole importance in the usually slow process of coagulation caused by *V cholerae* At the same time, carrying out seven successive series of tests with different kinds of milk, Schoffer noted a marked inconstancy of the coagulative reactions produced by most of his 14 cholera strains. He raised the question whether these inconstant findings, instead of being

[1] Lamas (1916) had previously established that two El Tor strains were capable of curdling milk within 9 and 20 days respectively while control strains of *V cholera* failed to do so even within 30 days.

25 cholera strains as well as a larger number of cholera like vibrios, mostly those isolated from surface waters, invariably produced acidity in litmus milk, usually within 24 hours of incubation at 37°C. Similarly Genevray & Bruneau, studying over 500 cholera strains in Indochina, found that these " invariably turned the colour of litmus milk into pink within 24-48 hours "

While, therefore, there is no valid reason to doubt that cultivation of *V cholerae* in milk leads to the production of an acid reaction to what extent this is followed by coagulation of the medium is a rather involved question

Referring to the initial observations made in this connexion by Koch and his co-workers in India, Gaffky (1887) considered it " most remarkable " that the cholera vibrios, though rapidly and abundantly multiplying in milk, did not produce coagulation or any other macroscopically observable reaction in this medium However as summarized by Schoffer (1895) soon afterwards some observers found that the strains at their disposal, which had been mostly isolated in Europe during 1892 and 1893 did coagulate milk. Indeed, this behaviour of the cholera strains derived from the 1892 epidemics in Paris and Hamburg led Liebreich (1893) to the assumption that these outbreaks had been caused by " comma bacilli " different from those isolated by Koch in India.

Though this postulation is now merely of historical interest, it is important to note that, while cholera strains capable of coagulating milk have practically never been met with in India, during the present century they have been detected upon several occasions in other areas The following observations may be quoted in this connexion

(*a*) Wherry (1905) found that one out of the five cholera strains he had recently obtained in the Philippines was capable of curdling milk within 48 hours.

(*b*) Examining the strains isolated during the Russian cholera epidemics of 1908-10 Buroff & Buroff (1911) noted that all these growths produced milk coagulation, which became manifest at 37°C on the second day of incubation, at room temperature after 10-12 days. It has to be added that the strains tested by these two workers were also unusual in so far as they proved to be endowed with haemolytic properties.

(*c*) Working in 1914 with 42 strains which had been isolated during the recent Balkan wars, Popoff Tcherkasky (1914) found that only five of these growths failed to curdle milk, while the others produced coagulation within 3-11 days. Most of the strains examined by this worker were also haemolytic.

(*d*) Pollitzer (1935), while finding that the cholera strains isolated in China during cholera epidemics produced no, or only late coagulation of milk, noted that two out of three strains isolated from sporadic cholera cases at Shanghai in 1934 as well as a strain agglutinable with cholera immune serum which had been isolated at the same time from the Whangpoo River combined the property of rapidly curdling milk with haemolytic properties. It is important to add that the strains of *V cholerae* isolated in China during epidemics proved non-haemolytic.

(*e*) Ali Mustapha (1936), comparing the behaviour of 37 cholera strains with that of 27 El Tor strains, recorded the following interesting results

when dealing with the practical aspects of cholera laboratory work ample use has been made of bile salt (sodium taurocholate) agar for the isolation of *V cholerae*

Blood media

The complex problem of the behaviour of *V cholerae* in fluid or on solid blood-containing media will be dealt with in a later part of the present chapter

Cultural variation

Kolle & Gotschlich (1903) who were the first to stress the occurrence of variant colonial types of *V cholerae* stated in this connexion that

"Petri was the first to point out precisely that in some cultures there occur besides typical, so-called atypical [colonies], which he called lobated colonies Later observations by Dönitz and Pfuhl showed that, if special substances, e g. asparagine, are added to the nutrient gelatin or if media with a low gelatin content (3 / 5) are used, the cholera colonies do not appear as round, brightly refractory discs with a slightly indented margin, which appear to be bestrewn with very small glass splinters, but show a yellowish coloration, coarser structure and an irregular margin, which sometimes looks frayed (formation of loops) as is common in old laboratory strains, which had been isolated during earlier epidemics.

"A careful examination undertaken in this respect with cultures recently arrived from Egypt in Berlin has left no doubt that in all fresh strains thus received, if they had been subcultivated once or several times one could invariably find both types of colonies which we might call transparent and opaque colonies." [Trans.]

Kolle & Gotschlich added that when subcultivating pure cultures on agar one also found two types of colonies those which were homogeneous and others which showed formation of a distinct rim or ring.

As confirmed by the exhaustive studies of Baerthlein (1911a 1912) the development of variant colonies took place not only on gelatin but also on agar plates, on which besides the above mentioned transparent colonies and ring colonies with an opaque centre and a transparent border yellowish white opaque colonies are apt to be present or even preponderant Baerthlein found that the transparent colonies were composed mainly of slender uniformly staining, and well-curved vibrios whereas microscopic preparations from opaque colonies revealed the presence either of short thick bipolar-stained vibrios or of longer well-curved forms showing instead of a uniform staining a segmental staining. It has to be noted however that in the experience of subsequent observers these morphological differences between the vibrios composing the transparent and opaque colonies respectively were not obligatory

While finding these two main colonial types remarkably stable if selectively subcultivated at frequent intervals, Baerthlein established that if growths displaying the presence of one of the types only were subcultivated after a lapse of time once more both colonial types became manifest

the result of a changing behaviour of the vibrios were not actually due to changes in the composition of the various kinds of milk used successively which it was impossible to assess Schoffer pointed out that this ever changing character of milk might, to quite some extent, explain the often contradictory statements made regarding the behaviour of *V cholerae* in this medium. However while the possible influence of such differences ought not to be disregarded the available evidence (see also Aida, 1939) seems to indicate nevertheless, the existence of a parallelism between milk coagulative and haemolytic properties of the vibrios, with the result that the El Tor vibrios are far more prone to curdle milk than the classical non-haemolytic cholera vibrios It would seem desirable further to elucidate this point by large scale parallel tests with cholera and El Tor vibrios, particularly in India where thus far but scanty attention seems to have been paid to the behaviour of these organisms in milk media.

Eggs

Notwithstanding statements to the contrary made by a few workers such as Hüppe (1888) Kolle & Prigge (1928) maintained that only slight growth of *V cholerae* takes place in eggs However the fact that Wilson (1946), infecting chick-embryos via the allantoic sac with cholera and cholera like vibrios noted a multiplication of the organisms not only in the allantoic fluid but also in the amniotic fluid and the egg yolk deserves attention. Moreover as pointed out by Kolle & Prigge the egg broth of Besredka & Jupille (1913) proved an excellent medium for the growth of cholera vibrios as well as for tuberculosis bacilli Similarly as will be described in a later chapter an alkaline egg-peptone medium has been recommended by Goldberger (1914) for selective enrichment in cholera diagnostic work. It also deserves attention that more recently Derkatsch (1927) utilized an alkalinized mixture of 150 ml egg yolk with 850 ml distilled water (pH 7 8) for the differentiation of *V cholerae* from " para-cholera " and other non cholera vibrios He claimed that true cholera vibrios reacted in this medium characteristically by producing within 42 to 72 hours incubation firm clotting of the substrate which was followed in 5-7 days by liquefaction with evidence of ammonia production.

Bile

It is of interest to add that Ottolenghi (1911) recommended an alkaline bile medium made with fresh cattle bile for the enrichment of *V cholerae* instead of peptone water Though Ottolenghi s method was considered useful by some subsequent workers, others, particularly Krombholz & Kulka (1912) found it less reliable than enrichment in peptone water In the opinion of Kolle & Prigge there was thus no reason to take practical advantage of Ottolenghi s method. However as will be further discussed

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While finding these two main colonial types remarkably stable if selectively subcultivated at frequent intervals Baerthlein established that if growths displaying the presence of one of the types only were subcultivated after a lapse of time once more both colonial types became manifest

Another point made by this worker was that recently isolated cholera cultures were more prone to show colonial variations than strains kept in the laboratory for a longer time This seemed to be explained by the observation of Haendel & Woithe (1910) that freshly isolated cholera growths were particularly sensitive to nutritional changes However other workers expressed the opinion that cultural variation was "more likely to occur in artificial cultures after continued growth on medium, but may be met with even in newly isolated strains" (Mackie 1929)

In a later paper Baerthlein (1918) distinguished between at least nine colonial variants of *V cholerae* but, as maintained by Gildemeister (1922) most of these seemed to present merely transitory or intermediate forms between the three principal types, the transparent, opaque and ring forms.

Examining seven cholera strains as well as two El Tor strains and one of "paracholera" vibrios Balteanu (1926) was able to distinguish between normal, round, translucent colonies and three variants, which he described as follows

(*a*) Circumvallate rugose colonies which were small, opaque, whitish yellow and had a thickened margin as well as radially arranged ridges. They were firmly adherent to the medium and could not be satisfactorily emulsified in saline or distilled water Transferred to broth, these growths produced a thick wrinkled surface film, which broke up on the tube being shaken and sank to the bottom, leaving the liquid clear Further reference to these rugose colonies will be made in the next section of this chapter

(*b*) White ring colonies, which were whitish and semitranslucent and sometimes had an opaque centre and more translucent margin thus resembling the ring forms of Baerthlein.

(*c*) Opaque colonies which were round, unusually prominent, firm in consistency adherent to the medium and difficult to emulsify Transferred to broth or peptone water these growths produced a thick, hard pellicle, resting on a clear medium, and a large deposit. While this colonial form proved stable on agar subculture repeated transfers in liquid media led to turbidity and gradual reversion so that, as described by Balteanu

"Plating from the 10th or 12th daily subculture yielded colonies with translucent margins and after further subcultures colonies of the normal type occurred."

Though Balteanu was able to procure this variant from four of his cholera strains and from one El Tor strain it was obtained regularly only from one of the former which, as it was found to be haemolytic for human and sheep corpuscles perhaps ought to have been placed in the El Tor group Studying this strain alone in detail, Balteanu found, as has been noted earlier in this chapter that the vibrios composing the opaque colonies possessed no flagella and were, in the opinion of the present writer for this reason, non motile Reference has also been made to the presence of a thick mucous envelope round the vibrios If the flagellar stain recommended

by Yokota (1924) was used the organisms in question were often uniformly and intensely stained but less frequently they showed granular or even bipolar staining

In consideration of these findings it is rather difficult to share the opinion of Balteanu that the unusual colonial form observed by him in an atypical strain was similar to the opaque variant described by Baerthlein More probably the appearance of the "opaque" colonies described by Balteanu was due to a process of *dissociation so that they represented the mucoid* (M) colonial type However in order to decide this point it would be certainly desirable to make in this respect further studies of the opaque colonies frequently met with in the course of cultivation of typical cholera strains

The significance of the above described cultural variations of *V cholerae* has been the subject of considerable debate Though it has been postulated in some quarters that they were the result of a true inheritable mutation there is every reason to share the opinion expressed by Mackie (1929) that these variants "do not however represent stable mutants but are to be regarded as fluctuating variations of the organism"

The observations of several workers that the appearance of colonial variants may be *promoted by artificially subjecting the cholera vibrios to* unfavourable influences e g to free chlorine or phenol (Genevray 1940a, 1940b 1940) serve as a corollary for the assumption that such variations are the result of a temporary adaptation and not of a permanent mutation.

It is however noteworthy that among the four types of cholera colonies distinguished by Husain & Burrows (1956) by cultivation on thionin glycerol agar only the "granular chromatic" type occurred on the plates used for the primary isolation of the causative organisms from the patients stools Hence it seemed probable to the two authors "that colonial types other than this may be regarded tentatively as variant types of uncertain relation to the human disease"

It *is important to note that colonial variation has been demonstrated* not only in the case of the classical *V cholerae* but also in the case of the El Tor vibrio (Balteanu, 1926 Alessi 1939) and as has been shown for instance by Feldmann (1917) and by Pasricha De Monte & Gupta (1932) likewise in the case of cholera like strains

Dissociation

Indispensable though it is to refer to the phenomena of dissociation at the present juncture, these processes being apt to exert a profound influence on the immunological properties of the *V cholerae* cannot now be fully appreciated More than that as shown for instance by recent observations on the dissociation of *V cholerae* by Bhaskaran (1953) a change in the growth appearances of this organism is by no means indispensable for indicating the presence of dissociation, which may often be demonstrated by biochemical or serological methods in cultures presenting no atypical

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Since the publication of this initial study the phenomena of S-R dissociation of *V. cholerae* have been exhaustively examined by different observers. The following of their findings have a bearing on the problems now under review.

Referring to the morphological appearances of the vibrios from smooth and rough cholera colonies respectively Seal (1937) maintained that

"the individual cell of the rough type has a more opaque and granular cytoplasm and a thicker outline than that of the smooth one which, on the other hand, possesses a clear cytoplasm and a thin wall. This may explain why the rough colonies usually look opaque and the smooth ones clear and translucent to the naked eye."

Panja (1945) who was able to produce rough colonies of *V. cholerae* by subcultivating smooth strains on agar into which mepacrine had been incorporated in the proportion of 1:5000 noted that in the case of the Inaba subtype some of the vibrios composing the rough colonies showed straight, spherical, or ovoid forms and were sometimes immotile. Ogawa rough vibrios showed long, straight besides typically curved forms but were invariably motile.

Further observations on the morphological differences between vibrios composing rough and smooth colonies respectively were recorded by Wahba (1953). In his experience "unlike other organisms the cholera vibrios in the rough state do not always appear in long or filamentous forms; on the contrary they might even be shorter and stouter than in the smooth state." Making impression films from well separated colonies, Wahba found the rough colonies to be composed of three zones: a central irregularly arranged core, an intermediate zone composed of radially lying organisms and peripherally outgrowing tufts composed of filamentous organisms. However, the outer zone was not filamentous in the case of some rough strains. Smooth cholera colonies appeared to possess two zones only, both of which were composed of radially arranged organisms.

Marked differences existing between smooth and rough cholera vibrios in regard to the mode of cell division and colony formation were described by Seal (1937) thus:

"The essential difference depends upon the degree to which the contiguous cells adhere to each other after undergoing division. The final cluster in a smooth culture is even in appearance owing to the cells sliding past each other and forming a smooth and compact mass, while in a rough culture the tendency to slip past each other is almost absent and the cells after division tend to adhere to each other more firmly leading to the formation of bending and branching chains and irregular masses with many open spaces, projections, angles and sometimes chains, sticking out from their sides, the final cluster being thus jagged and uneven in appearance."

The differences existing between smooth and rough growths of the *V. cholerae* in fluid media were in Seal's opinion due to identical causes. In the case of the smooth type, cultivation in broth or peptone water

features as far as their macroscopic aspect is concerned. On the other hand, it is not surprising to find that, though the presence of macroscopically characteristic dissociants had been noted by some early workers the occurrence of such atypical colonies was confused with that of colonial variants which, as has been described resulted from an adaptation of the cholera vibrios to unfavourable environmental conditions

Hadley (1927) dealing comprehensively with the early observations of dissociation in the various bacterial species, mentioned a few records dating back to 1894 which, in his opinion, referred to dissocants of *V cholerae* It would seem, however that Berestneff (1908) was the first who definitely noted the rough form of this organism, stating

"that cholera vibrios, if repeatedly transferred from agar to agar sometimes begin to grow in the form of dry prominent and non-confluent colonies. Many such colonies show a crater-like depression and a wall-like periphery Such colonies are markedly different from the normal ones on account of their dryness they are difficult to emulsify and show pseudo-agglutination, being thus unfit for agglutination tests."

Though there can be little doubt that Berestneff referred to the rough form of *V cholerae* it was only after the pioneer studies on microbic dissociation had been published by Arkwright (1921) and De Kruif (1921) that Shousha (1924) gave a full description of the properties shown by the smooth (S) and rough (R) types of this organism respectively

Shousha worked with two old cholera laboratory strains one of which proved to be haemolytic when tested with sheep erythrocytes Both were inoculated into broth tubes which, after an incubation at 37°C for 24 hours were stored at room temperature in the dark When agar subcultures were made after such storage for 15 days the haemolytic strain only showed two types of colonies similar to the S and R forms described by Arkwright (1921) in salmonellae.

While not referring to the morphological appearances of the vibrios composing these two types of colonies respectively Shousha stated that both were equally motile The differences in growth appearances observed by him may thus be summarized

Medium	*S type*	*R type*
Agar	Colonies circular with well-defined margins, finely granular under low power of the microscope.	Colonies larger flat and thin, appear granular with jagged margins when seen under low power of the microscope.
Broth or peptone water	General turbidity and pellicle formation.	Deposit at bottom and pellicle formation, the body of the fluid remaining clear *

* Uniformly turbid growth was obtained either if media prepared with less salt were used or if the usual media were diluted to one-half or one-quarter with distilled water

Polysaccharides

Form	Dominant	Also present
Smooth (S)	C α	C β, C γ C δ
Rough (R)	C β	C γ C δ
Rho (ρ)	C δ	C γ

Bruce White added that though no variant degraded below the level of the ρ form had been discovered such forms possibly existed Carrying out comparative studies he found that

"the true El Tor vibrio presents a polysaccharide complex serologically identical with that of V cholerae (of the same absorption type) that the C γ and C δ factors seem to be common, so far as can be judged by simple precipitation tests to all the types of vibrios so far examined that different groups of vibrios show sharp differences in the behaviour of their C β substances and that the C α substances determine the serological specificity of the various smooth types"

Besides elucidating the phenomena of S-R dissociation of *V cholerae* Bruce White (1938, 1940) also dealt in a masterly manner with the rugose form of this organism He stated in the latter connexion that Balteanu (1926) working with cholera and El Tor vibrios had noted the occurrence of a variant colonial form which he designated as rugose on account of its corrugated appearance on agar However Bruce White added

"From a detailed description of the rugose variant Balteanu was probably deflected by the fact that it proved unstable in culture his attention was occupied with a more stable opaque variant which is perhaps a cultural form much of the same genre."

This is in accordance with what has been stated above in regard to Balteanu's "opaque" form

Even though, as shown in Table XVIII the appearances and properties of rugose growths are markedly different from those of the rough forms of *V cholerae* some workers were inclined to regard the rugose variant as the extreme type of roughness It is the great merit of Bruce White to have pointed out that actually a fundamental difference exists between the two modes of dissociation concerned while, as described above the process of roughening is due to a failure to secrete or to form specific polysaccharides rugosity is the result of an abnormally active secretion of a mucinous material, ascribed by Bruce White to an intensification of normal secretory processes rather than to a special type of activity In fact, a transition to the rugose state could be observed in the case of rough or even ρ growths as well as in smooth growths of cholera vibrios and particularly of El Tor vibrios The marked tendency of the rugose derivatives to return to their original state (S R, or ρ) as contrasted with the stability of the ordinary R form also rendered it altogether improbable that rugosity represented a culmination of roughness

Though observed in growths from cholera stools, the rugose type of colonies was particularly met with in platings from aging peptone water

resulted in a marked and uniform turbidity with or without a thin pellicle, because the vibrios did not tend to stick together after division. The rough strains on the contrary, formed chains and the irregular clusters thus resulting led to the formation of a thick pellicle on the surface of the fluids as well as to the formation of granules which as a rule sank down but could remain partially suspended then producing a slight turbidity of the media.

Observations made by Soru (1934) with 106 cholera and cholera like vibrios showed that vibrios of the R type had a higher negative electric charge than those of the S type These results were confirmed by Linton Mitra & Seal (1938), who partly examined dissociants they had obtained from Bruce White. Linton and co-authors noted the interesting fact

" that electrophoretically the organisms which are quite distinct from one another in the S state are often similar or identical in the R state. This is perhaps the underlying factor to account for the observation of Bruce White who found R strains serologically more generalized than the S strains. In the case of Shillong 1077 the ρ strain showed an even higher surface potential than the rough homologue and was very much higher than the original smooth strain."

The studies of Bruce White referred to above led to a full understanding of the phenomena underlying dissociation of the *V cholerae* by furnishing evidence to prove the contention made in a preliminary statement by Yang & White (1934) that in case of *V cholerae* as in that of the salmonellae and the pneumococci " roughening involves the disappearance of a non protein and probably carbohydrate containing substance which furnishes the characteristic O-receptor of the smooth organism " " It seems certain," Yang & White continued, " that a second non-protein substance, present but masked in the smooth organism, replaces in the rough vibrio the lost smooth factor and becomes the characteristic rough receptor "

In a further paper published in 1934 Bruce White established that in the case of *V cholerae* as well as in that of the salmonella group a ρ variant existed which differed from the R form by loss of the dominant R receptors Bruce White added that according to various tests

" this loss involves the bulk of those receptors which are supplied by the alkali-resistant, non-protein and richly carbohydrate soluble substance of the rough vibrio "

Continuing his studies Bruce White (1936) was able to establish that " in each strain of *V cholerae* and seemingly in vibrios in general, at least four distinct groups of polysaccharide receptors or substances are concerned in the serology of the normal parent and variant forms " All four of these substances, named C α, C β C γ and C δ, were present in the smooth form but C α was dominant In the rough form, C α was absent and C β was dominant, whereas only C γ and C δ were present, and the latter was dominant, in the ρ form. The distribution of these four poly saccharides may therefore, be schematized thus

strains whereas, as will be discussed later, the stable degradation brought about by transition into the rough state is instrumental in rendering the vibrios concerned non infective

Though the occurrence of a third type of dissociation of *V cholerae* leading to the growth in the form of minute pleuropneumonia like (L) colonies has been established through recent observations only (Minck 1950 1951 Minck & Minck, 1951 , Carrère & Roux 1953), the presence of dwarf colonies has been recorded by some earlier workers first apparently by Baerthlein & Grünbaum (1916)

These two workers stated that some diagnostic difficulties were caused by the occurrence of minute colonies, reaching hardly pin-point size within 24 hours of incubation even on Dieudonné agar which often developed alone on the plates used for isolation of *V cholerae* Smears from such growths showed the presence of very slender vibrios which, as they formed chains, resembled relapsing fever spirochaetes. Subcultivation on solid media did not lead to a change in the growth appearance of such strains. However if the vibrios were kept for some time in broth and then subcultured on suitable media, typical, opaque and ring colonies developed.

Minck & Minck (1951—see also Minck 1950, 1951) were able to produce L-dissociation of *V cholerae* through subcultivation of primary cultures from intraperitoneally infected mice on soft serum agar containing 1000 units of penicillin per ml but had no success with stock cultures

After an incubation of the penicillin-containing cultures for six to eight hours, dwarf colonies with a maximal diameter of 500µ became visible. Their centre was found to consist mainly of minute L forms (" elementary bodies ") while on the periphery giant globular bodies (diameter 10-20µ) were seen which were more or less filled with motile granules. Intermediate forms were likewise encountered.

It was possible to maintain the strains in this dissociated condition by weekly subculture on penicillin-serum agar Transfers from these subcultures to ordinary serum-agar led at first to the development of normal cholera colonies, but after five to six passages through penicillin-containing media, dwarf colonies developed even on ordinary serum-agar and proved stable upon subcultivation on the latter medium.

Intraperitoneal inoculation of mice with little-subcultivated L growths led to no pathological changes but seemed to confer a certain degree of immunity against infection with normal cholera vibrios. Inoculation with " fixed " L growths (i.e., those showing no tendency to revert to type on ordinary serum-agar) often led to the death of the animals. Autopsy showed the presence of acute peritonitis and necrotic enteritis. On two occasions only a few L colonies developed in cultures from the peritoneal exudate, while, as a rule, enteric bacteria alone seemed to be present.

The findings of Carrère & Roux (1953) were on the whole similar to those described above It is noteworthy however that according to their observations (*a*) a stock Inaba strain of *V cholerae* was found to produce numerous L forms and globular bodies in ordinary broth (*b*) L forms developed on semi solid media inoculated with the filtrate of a five-day-old dissociant peptone water culture through an L_3 Chamberland candle and (*c*) the L forms appeared to be non pathogenic for white mice while percutaneous or subcutaneous as well as intraperitoneal inoculations with

TABLE XVIII. CHARACTERISTICS OF S R AND RUGOSE GROWTHS OF V CHOLERAE

	Normal S culture	Typical R culture	Rugose culture derived from S culture
Appearance of colonies on agar plates.	Circular moderately raised glistening and moist finely granular under lens and of variable transparence or turbidity Occasionally colonies may show features usually associated with roughness.	As a rule differs but slightly in general appearance from S colony so that it cannot be identified with certainty by simple inspection. Usually duller of surface and more coarsely granular may show irregular outline and flattening Outward appearances not indicative of intensity or permanence of roughness.	The 18 hours colony is small much raised and refractile. It increases rapidly with further incubation and develops superficial corrugation, irregular radial, or both. Opaque yellowish or yellow in colour opacity and tint deepening with age. In older colonies vitreous or granular " corona " may be exuded from margin of the colony
Consistence and adherence.	Semi-fluid, never adherent to medium.	Dry brittle never adherent.	Tough or gelatinous, adherent to medium.
Dispersability and agglutination in NaCl solution.	Disperses readily with perhaps some initial sliminess. Dense suspensions in 1 7% NaCl solution may show some precipitation of slime entrapping a few vibrios.	Disperses readily in 0 85% or 1 7% NaCl solution but complete agglutination follows quickly so that clumps float in clear liquid	Growth disperses only partially and with difficulty Vibrios once dispersed are insensitive to saline.

After Bruce White (1938)

cultures, and it appeared that higher peptone concentrations (5% 10%) favoured this mode of growth. More important still rugose colonies were found to abound in platings of vibrios which had survived specific bactericidal tests

Evaluating these observations, Bruce White (1940) stated

" It is difficult to escape the conclusion that the rugose substance is a protective secretion with a role in assisting the survival of the race in nature. In the laboratory it affords defense against unfavourable conditions and the action of serum It has repeatedly been observed that rugose forms tend to grow often in pure culture from mixtures made in specific bactericidal tests and to survive their associates in ageing cultures of vibrios."

The rugose variants did not, however prove more resistant than normal cholera vibrios when kept in saline solutions or grown in broth to which hydrochloric acid had been added, so that there was no reason to assume that they would have a particularly good chance of resisting the acid conditions in the human stomach. Nevertheless, the evidence that they are more resistant than non rugose vibrios is convincing. Considering this as well as the marked tendency of rugose growths to revert to type one might look upon rugose transition as a means of prolonging the life of infective cholera

strains whereas as will be discussed later, the stable degradation brought about by transition into the rough state is instrumental in rendering the vibrios concerned non infective

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Minck & Minck (1951—see also Minck 1950 1951) were able to produce L-dissociation of *V cholerae* through subcultivation of primary cultures from intraperitoneally infected mice on soft serum agar containing 1000 units of penicillin per ml but had no success with stock cultures.

After an incubation of the penicillin-containing cultures for six to eight hours, dwarf colonies with a maximal diameter of 500μ became visible. Their centre was found to consist mainly of minute L forms (" elementary bodies "), while on the periphery giant globular bodies (diameter 10-20μ) were seen which were more or less filled with motile granules. Intermediate forms were likewise encountered.

It was possible to maintain the strains in this dissociated condition by weekly sub-culture on penicillin-serum agar Transfers from these subcultures to ordinary serum-agar led at first to the development of normal cholera colonies, but after five to six passages through penicillin-containing media, dwarf colonies developed even on ordinary serum agar and proved stable upon subcultivation on the latter medium.

Intraperitoneal inoculation of mice with little-subcultivated L growths led to no patho-logical changes but seemed to confer a certain degree of immunity against infection with normal cholera vibrios. Inoculation with " fixed " L growths (i.e., those showing no tendency to revert to type on ordinary serum-agar) often led to the death of the animals. Autopsy showed the presence of acute peritonitis and necrotic enteritis. On two occasions only a few L colonies developed in cultures from the peritoneal exudate while, as a rule, enteric bacteria alone seemed to be present.

The findings of Carrère & Roux (1953) were on the whole similar to those described above It is noteworthy however that according to their observations (*a*) a stock Inaba strain of *V cholerae* was found to produce numerous L forms and globular bodies in ordinary broth (*b*) L forms de-veloped on semi solid media inoculated with the filtrate of a five-day-old dissociant peptone water culture through an L_3 Chamberland candle and (*c*) the L forms appeared to be non pathogenic for white mice while per cutaneous or subcutaneous as well as intraperitoneal inoculations with

material containing globular bodies killed these mice in 24 hours Carrère & Roux were inclined to assume that the globular bodies might be a resistant form of *V cholerae*

Biochemical Properties

Specific chemical constituents

Though Galeotti (1912) claimed to have isolated a nucleoproteid of *V cholerae* as early as in 1896 and Landsteiner & Levine (1926) laid a firm foundation for future work by extracting from a cholera strain a carbohydrate-containing substance which reacted specifically with cholera immune serum, it was only within the last two decades that systematic studies on the immunochemistry of the cholera vibrio have been carried out by Linton and his co-workers as well as by some other investigators particularly Bruce White whose observations will, however be considered in the following chapter

Linton and his co-workers (see Linton Shrivastava & Mitra, 1935 Linton 1940 1942) observing the optical activity of the proteins of numerous cholera and cholera like strains in dilute alkali solutions were able to distinguish between two types of protein They also found that three types of polysaccharides occurred in these vibrios, composed respectively of (1) galactose and an aldobionic acid consisting of galactose and glycuronic acid (2) arabinose and an aldobionic acid identical with that of group (1) (3) glucose only It was thus possible to class the cholera and cholera like vibrios into six groups composed as follows

Number	Protein type	Polysaccharide type	Nature of vibrios composing group
I	I	I	Majority of true cholera strains, occasionally water vibrios.
II	I	II	Cholera strains, specially those from Assam (rare in Calcutta), occasionally water vibrios.
III	II	II	Mainly water vibrios, not agglutinable with cholera immune serum.
IV	II	I	El Tor strains and some identical strains isolated from carriers in India.
V	II	III	Cholera strains from carriers.
VI	I	III	Rare in nature, mainly found in old laboratory strains of *V cholerae*

As will be noted, even apart from the tediousness of the procedures involved, it would not be possible to make with the aid of these tests a diagnostically valid distinction between cholera and cholera like vibrios. It is, however interesting to see that the El Tor vibrios, though showing in most respects features identical with those of the classical non haemolytic cholera vibrios, fell according to the above method of classification into a separate group

Linton Mitra & Mullick (1936) studying the respiration and glycolysis of cholera and cholera like vibrios found metabolism to be most active when the organisms belonged to Group I less so in the case of Groups II V and VI least active in case of the Group III vibrios Group IV to which the El Tor vibrios belonged was according to these workers " sharply marked off " by the absence of glycolysis under aerobic conditions of growth Rough dissociants showed a lower metabolism than their smooth parent strains

The observation that vibrios of Group I to which most true cholera vibrios belonged was metabolically most active is in accord with Bernheim s observation (1943) that *V cholerae* had 24% more reactive amino groups than *E coli* Since then the presence of hitherto unknown amino-acids of the cholera vibrio has been recorded (Blass & Macheboeuf 1945 1947, Blass et al 1951 Banerjee Roy & Ganguli 1956, Blass 1956)

Enzymatic make-up

Proteolytic enzymes

As far as could be ascertained Bitter (1886) was the first to establish that the liquefaction of gelatin-containing media by *V cholerae* was due to the presence of a proteolytic enzyme or as he called it "ferment" which exerted an influence analogous to that of trypsin Wherry (1905) summarizing further early observations made in this direction stated that in analogy with the behaviour of trypsin, the proteolytic enzymes of the cholera vibrio as well as of other bacteria were operative only in alkaline media, the presence of even small amounts of acids hindering their action The presence of fermentable carbohydrates in the media was found to inhibit the liquefaction of gelatin but it deserves attention that according to Auerbach (1897) who though devoting some attention to *V cholerae* experimented mainly with *Proteus vulgaris* this absence of liquefaction was due not to the appearance of acids in the course of cultivation but to an inhibition of the formation of the proteolytic enzymes Besides the presence of protein substances in the media, the access of free oxygen was found to be essential for the production of these enzymes, liquefaction of gelatin taking place very slowly if at all, under anaerobic conditions (Liborius 1886)

Summing up his own observations, Wherry stated that the type of liquefaction was influenced to a marked degree by the melting point as well as the reaction of the gelatin used, and added that

> the optimum condition for growth is furnished by an albuminous medium containing between 1/50 and 1/100 gram-molecule of NaOH or Na_2CO_3 per liter and this corresponds fairly well with the optimum conditions for the tryptic digest of fibrin "

In a recently published study Agarwala & Shrivastava (1953) recorded the results of viscosimeter measurements of the "gelatinase" activity of

cholera and cholera like strains grown for 24 hours in papain broth to which gelatin at a concentration of 5% had been added They found that growths of cholera like water vibrios displayed a 25% greater gelatinase activity than all other strains tested which included, besides true cholera vibrios also those of the El Tor group The pH optimum for the gelatinase activity of both true cholera and water vibrios was found to be about 8 0 Incubation of the cultures for longer periods failed to increase the activity of the enzyme which was found to be stable for a long time in growths kept at 37°C.

As recently stated by Nihoul (1952) the presence of calcium exerted an impeding effect on the proteolytic activity of *V cholerae*

While, as apparently first demonstrated by Beaujean (1913) and generally accepted, no correlation exists between the proteolytic and the *haemolytic* properties of the cholera vibrios, the question of the relationship between the former property and the *haemodigestive* action of these organisms referred to later in this chapter has been the subject of debate Bernard, Guillerm & Gallut (1937a 1937b) reached the conclusion possibly arrived at earlier by Loewy (1915) that the proteolytic and haemodigestive properties of the *V cholerae* were due to the action of one and the same enzyme but Beeuwkes (1939) adduced evidence to show that probably different enzymes were responsible for the gelatin liquefaction and haemodigestion respectively produced by cholera and El Tor vibrios

Milk-coagulating enzyme

As has been discussed above (see page 123) the production by *V cholerae* of an enzyme identical in action to that of rennet, which was capable of coagulating milk even in the presence of a weakly acid reaction, has been demonstrated by Fokker (1892)

Collagenase

Studying the action of culture filtrates of *V cholerae* on pure collagen prepared from buffalo tendons Narayanan & Menon (1952) stated that they had demonstrated the presence of a collagenase This enzyme which was found capable of acting over a wide pH range with an optimum at pH 8.0 was probably also present to some extent in the culture filtrates of cholera like vibrios, but could not be demonstrated in those of *E. coli*

Elastinase

In a further study published in 1953 Narayanan Devi & Menon reported on the presence of an elastinase, active against elastin prepared from buffalo ligaments in the cultures of two out of the 7 cholera strains examined in this respect. The presence of this enzyme as well as that of collagenase was also demonstrated in cultures of cholera like vibrios

Lecithinase

Reporting in 1944 on the lecithinase activity of *V cholerae*, Felsenfeld referred to earlier studies made in this respect by Ruata & Caneva (1901) and Kraaij & Wolff (1923) While the latter workers demonstrated the presence of lecithinase only in El Tor vibrios Ruata & Caneva found this enzyme to be present in all vibrio strains examined by them Felsenfeld s investigations also showed lecithinase activity in four true cholera strains as well as in one El Tor strain The optimal temperature for the action of the enzyme was 36°-38°C, the optimal pH 7 4-7 6 Lecithinase activity was stimulated by calcium and magnesium, whereas formaldehyde phenol and fat-soluble narcotics exerted an inhibitory effect

Deaminases

As recorded by Dudani et al in 1952 (see also Iyer et al, 1953 1954 Iyer & Krishna Murti 1955) *V cholerae* possess deaminases in their enzyme make up the rate of deamination varying from one amino-acid to another and differing in different strains Among the amino-acids studied deamination of aspartic acid and serine was maximal but arginine, glycine glutamic acid lysin and threonine were also deaminated Deamination took place under strictly aerobic conditions only and was optimal at a pH range of 7 0 to 8 0 It is interesting that in general cholera vibrios of the Ogawa subtype showed a higher deaminase activity than those of the Inaba subtype

Further investigations by Arora and colleagues (1956) showed that sodium chloride exerted a stimulating action on the production of certain deaminases by the cholera vibrio

Studying the metabolism of purine and pyrimidine compounds of a few strains of *V cholerae* and other vibrios Agarwala et al (1954) found that deamination appeared to be the only active process in the utilization of purine nitrogen

Nucleotidase

According to observations by Krishna Murti & Shrivastava (1955) *V cholerae* and cholera like vibrios while possessing no phosphatase activity were endowed with nucleotidase activity and were thus capable of readily attacking the phosphoric acid ester linkage of purine and pyrimidine compounds.

Dehydrogenases

Dudani et al in 1953 published the results of preliminary observations on the dehydrogenation of various substrates by an Ogawa strain of *V cholerae* and the Inaba and rough variants derived from it. Almost all amino-acids and aliphatic acids employed in this study were found to

be capable of acting as hydrogen donors for the respiratory activity of the organism The dehydrogenases of *V cholerae* seemed to be linked up with the cytochrome systems present in this organism, but the possibility of other coenzyme systems taking a part in the process of dehydrogenation could not be excluded.

γ-peptidase As stated in a preliminary report, Agarwala et al. (1953b) studying the hydrolysation of glutathione by *V cholerae* found evidence pointing to the probable presence of γ-peptidase in the cells of this organism.

Mucinase and tissue-disintegrating enzyme

The important studies of Burnet (1948 1949) and Burnet and co-workers (1946 1947) on the enzymes of *V cholerae* go back to investigations made by Burnet, McCrea & Stone in 1946 on the receptors of human red blood corpuscles for virus action. These workers found that red cells which had been treated with influenza virus while losing their agglutinability by some or all of the viruses of the mumps-influenza group developed an agglutinability with almost any human serum (" pan-agglutinability " of Thomsen, 1926) Since Friedenreich (1928) had shown that such a pan agglutinability with sera of all blood groups and even with that of the donor of the red cells could be produced with the aid of cholera and cholera like vibrios Burnet McCrea & Stone experimented with the culture filtrates of two cholera and one cholera like strain They found that an action almost completely analogous to that of the above-mentioned viruses, and due undoubtedly to enzyme activities could be produced with these culture filtrates.

Following up this work, Burnet & Stone (1947) made tests with various substrates to explore the possibility that the receptor-destroying enzyme of the filtrates of cholera cultures was a collagenase The important result of these studies was the demonstration of an actively desquamating effect exerted by these filtrates on the intestinal mucosa of guinea pigs and rabbits. As stated by Burnet & Stone

" it soon became evident that this action was not a function of the receptor-destroying enzyme but the possible relationship of such *in vitro* desquamation to the pathogenesis of human cholera seemed to justify an independent investigation of the phenomenon "

Burnet & Stone summarized the results of this investigation thus

" (*a*) Filtrates from *Vibrio cholerae* cultures are capable of producing desquamation of the intestinal epithelium *in vitro*

" (*b*) There is a well-marked gradient of diminishing susceptibility to desquamation from the jejunum to the descending colon

" (*c*) Histological and other preliminary evidence suggests that the principal agent concerned is a mucinase

" (*d*) Intestinal mucin is rapidly dissolved by active filtrates, the effect paralleling the desquamation reaction both are similarly neutralized by rabbit immune serum (prepared with the aid of *V cholerae* culture filtrates)

" (*e*) Evidence is given for the existence of another enzyme concerned with breaking down the cement substance between cells."

Discussing the importance of these findings Burnet & Stone pointed out that

"the mucinase described in this paper can rapidly destroy the viscosity and hence the mechanical protective and lubricating properties of intestinal mucus. Experiments in progress show that this action can take place in isolated gut segments in the living animal and if the enzyme, as seems likely is produced in large amount in the bowel of a cholera patient, it might well play a major part in facilitating desquamation of the intestinal epithelium. The third agent (i e. the tissue-disintegrating enzyme) on which very little work has so far been done, by breaking down some presumed components of the cement substance between cells would also favour the desquamating process."

No evidence was obtained to show that the receptor-destroying enzyme played a role in this process of desquamation and tissue-disintegration

Reporting on further studies of the cholera mucinase Burnet (1948) stated that this enzyme was found to be active against a variety of glandular mucins but exerted no action on human synovial fluids (hyaluronic acid type mucin) The activity of mucinase was found to be completely inhibited by sodium hexametaphosphate and (like hyaluronidases) it was inert in the absence of salts

As recorded by Burnet in a further communication published in 1949, it had been found possible to treat the vibrio filtrates so that they contained either mucinase or the receptor-destroying enzyme alone in active form if the filtrates were treated with an excess of $CaCl_2$, brought to a pH of 6 and heated for 30 minutes at 56°C the mucinase alone was destroyed However if the filtrates were alkalinized to pH 8 5 and held for some hours at 37°C, the mucinase remained fully active while the receptor-destroying enzyme was totally inactivated

Publishing further observations on the intestinal-epithelium-destroying enzyme Singh & Ahuja (1953) stated that they could demonstrate its presence not only in all smooth cholera strains but also in most El Tor and cholera like strains examined by them These two workers concluded therefore,

"that mucinase activity is not specifically confined to *V cholerae* but is shared by other members of the genus vibrio Whether or not this enzyme plays any role in the causation of [the] cholera syndrome is a moot point."

Making a study of the serological character of the mucinase of *V cholerae* and other vibrios Freter (1955b) established in analogy with the views of Singh & Ahuja that three strains of "non agglutinating" water vibrios isolated in Chicago produced high titre mucinases which in two cases were serologically related to the enzymes of the cholera vibrios. However, while maintaining that apparently "the titer and serological type of mucinase produced in vitro has no relation to virulence colonial morphology or O antigenic structure of the tested cholera strains" Freter was careful to point out that "the data presented do of course, not give information as to the actual relation of mucinase or other enzymes to the pathogenicity of cholera vibrios"

In regard to the latter point it deserves attention that (1) Freter (1955a) was able to demonstrate mucinase in the bowel fluid of guinea pigs which had succumbed to enteric cholera infection and (2) Lam, Mandle & Goodner (1955) adduced experimental evidence that

"*V comma* mucinase (or the mucinase complex of enzymes) alters the permeability of the mouse intestine but that this effect is blocked by immunization against mucinase both by passive and active procedures."

Penicillinase

Investigations by Iyer Dudani & Krishna Murti (1954) indicated that cholera and El Tor vibrios did not exhibit any penicillinase activity and that consequently the low susceptibility of *V cholerae* to penicillin could not be due to the elaboration of such an enzyme by the organisms. Four among the nine strains of cholera like vibrios tested were found to produce penicillinase

Decarboxylase

According to Ogasawara & Kariya (1954) cholera vibrios were capable of producing at an acid pH a lysine decarboxylase enzyme which converted lysine to cadaverine (pentamethylene diamine) The action of this enzyme probably accounted for the presence of cadaverine in the stools of cholera patients

Lipase

The production of a lipase active against olive oil by *V cholerae* as well as by cholera like vibrios has been recently demonstrated by Narayanan Devi & Menon (1953)

Carbohydrate-converting enzymes

Bitter (1886) seems to have first drawn attention to the amylolytic activity of the *V cholerae* due to the action of "ferments" (enzymes) His observations were soon confirmed by several other workers (see Nobechi, 1925) Wherry (1905) one of the pioneers in this field, stated that all six cholera strains examined by him produced not only amylase and maltase (as had been previously found to be the case by Buxton, 1903) as well as invertase (already found by Sclavo 1892) but also lactase

Indole formation

As will be described in a later chapter application of Ehrlich s rosindole test shows that cholera vibrios if suitably cultivated, invariably produce indole. Since however other intestinal bacteria as well as many of the cholera like vibrio species also react positively in this respect, such tests have no differential diagnostic importance

As summarized by Sticker (1912) Hoppe-Seyler (1892) found that indole accumulates in the intestine of cholera patients because it is no longer destroyed by oxidation as in the healthy body The large amounts of indican and indoxyl sulfuric acid found in the urine of cholera patients also indicated according to Hoppe Seyler an increased indole production

Nitroso-indole (cholera red) reaction

It is curious to note that tests based on the phenomena underlying the nitroso-indole or, as it is commonly called the cholera red reaction carried out after 1883 with cholera cultures, had been utilized well before that year with the aid of the dejecta of cholera patients According to Sticker (1912) Kopp (1837) was the first to observe that addition of small amounts of pure nitric acid to cholera stools or their distillates produced a red colour and similar results were recorded by some subsequent observers including Virchow (see Schuchardt 1887), who partly used other mineral acids.

After Koch had isolated *V cholerae* the testing of suspicious cultures with mineral acids so as to determine whether or not the red coloration considered characteristic for this organism appeared was recommended by Poehl (1886) and independently by Bujwid (1887) and Dunham (1887) Bujwid emphasized in his short note which appeared before Dunham s article, the importance of the method for a rapid diagnosis of cholera since a pink to reddish violet colour appeared quickly when a few drops of 5% to 10% hydrochloric acid had been added to broth cultures of cholera vibrios grown at 37°C for 10-12 hours He considered the test as practically specific for *V cholerae*

Recording the results of an investigation into the phenomena underlying this test, Salkowski (1887) stated with admirable clearness and brevity that the "cholera reaction" is

"nothing else than a quite common indole reaction, and the explanation for the fact that the indole reaction can be produced in cholera cultures with sulfuric acid alone is simply that the cholera vibrios constantly produce nitrous acid, which is present in the fluid in the form of nitrites There exists no specific cholera red, as has been assumed by Brieger this is simple indole-red and demonstrable in every decomposing peptone solution. Characteristic of the cholera bacteria is only the *simultaneous* production of indole and nitrous acid." [Trans.]

The validity of Salkowski s statement that the cholera red reaction is due to the ability of the cholera vibrio of reducing nitrates to nitrites as well as to the production of indole by this organism has been generally accepted

The technique of the nitroso-indole test will be duly described in a later chapter It has to be noted, however that, whereas the early workers considered this reaction one of the principal methods or even the cardinal method of establishing the presence of *V cholerae* it has now hardly any importance in practical cholera laboratory work. For it has been

established that on the one hand positive reactions are also produced by certain cholera like vibrios and even by bacteria belonging to other genera, while on the other hand for reasons which will be specified when dealing with the problems of cholera diagnosis false negatives may be obtained even though *V cholerae* is present. However as will be noted later Taylor Pandit & Read (1937) ascribed some usefulness to the cholera red test, if used in combination with other biochemical methods.

Saccharolytic effects

While as shown by the tabulation below *V cholerae* has been found capable of causing acidification of media into which certain carbohydrates had been incorporated, it has to be emphasized that this process is never accompanied by the formation of gas

Though the whole of the available litera ure has been considered for compilation of the tabulation it is based mainly upon data furnished by Heiberg (1934) because this worker used a satisfactory modern technique (*a*) growing the strains to be tested in peptone water (pH 8 0 – 8 4) into which the various carbohydrates had been incorporated at a concen ration of 0 5 % (*b*) adding bromothymol as indicator in place of litmus (which—as first shown by Müller (1899)—is apt eventually to become reduced by *V cholerae*) and (*c*) taking initial readings not later than after an incubation of 20 hours at 37°C so as to be able to distinguish between rapid and late acidification

Reactions produced by Vibrio cholerae *in Carbohydrate-containing Media*

Constant and rapid acidification	*Late acidification*	*No change*
Arbutin (Pergola, 1921)	Glycerol [d]	Adonitol
Dextrin	Lactose [d]	Arabinose [c]
Erythritol (Violle, 1919)		Dulcitol
Galactose		Inulin
Glucose		Inositol
Glycogen		Rhamnose
Levulose		Salicin
Mannose		Sorbite
Maltose		Tartrate
Mannitol [b]		Xylose
Saccharose		
Starch		

Late acidification according to Heiberg (1934).
[b] Negative in eight out of nine strains according to Noury & Alalou (1923) variable according to Seal (1935)
[c] See text.
[d] Variable according to some workers.
[e] Variable according to Seal (1935)

As stated by Kauffmann (1934) when reporting upon the observations of Heiberg (1934) referred to later the reactions produced by individual

cholera strains in carbohydrate-containing media are stable as proved by re-examination of cultures which had been kept in the laboratory for periods varying from six months to one year Identical findings have also been recorded by some other workers but the following statements must be noted

(*a*) According to observations by Mesnard & Genevray (1931) cholera vibrios which grew on account of variation in the form of opaque colonies with a wrinkled surface produced more vigorous acidification of glucose and saccharose than the parent strains

(*b*) Seal (1935) maintained that in general the saccharolytic effects of cholera strains could undergo changes in the course of subcultivation probably hand in hand with variation of the organisms themselves, a dissociant of a typical smooth strain in particular producing some acidification only in the presence of glucose

An elaborate attempt to use tests with carbohydrate-containing media for the classification of cholera and cholera like vibrios was made by Heiberg (1934) who established that it was sufficient to use three substances only namely saccharose arabinose and mannose for this purpose. The results obtained in this manner by Heiberg were thus summarized by Kauffmann (1934) in the *Bulletin de l Office international d'Hygiène publique*

Group	*Saccharose*	*Arabinose*	*Mannose*	*Strains agglutinating with cholera-immune serum*	*Strains not agglutinating with specific serum*	*Spontaneously agglutinating strains*
I	+	0	+	239	27	13
II	+	0	0	1	76	0
III	+	+	+	0	12	1
IV	+	+	0	0	3	0
V	0	0	+	0	2	0
VI	0	0	0	0	5	0

It will be noted that, with the doubtful exception of one atypical and weakly agglutinating strain the true cholera vibrios fell in Heiberg s Group I The vibrios not agglutinating with cholera immune serum, on the contrary, fell in all six groups, far more in Groups II and III than in Group I which seemed thus a class rather characteristic of *V cholerae*

Workers in the cholera areas of India and China soon confirmed that practically always the true cholera vibrios belonged to Heiberg s Group I In fact, the only observers recording some aberrant results were Seal (1935) in India, and Yu (1938) in China

Seal maintained that (*a*) some cholera strains isolated from carriers failed to produce acidity in saccharose-containing media, and (*b*) arabinose was affected by a very small percentage of cholera vibrios.

Yu in 1938, examining 52 smooth cholera strains which had been isolated during the Shanghai epidemics of 1932 and 1937 found evidence of late arabinose fermentation in some instances, and noted that three of the 1937 strains failed to acidify mannose and

—if the present author may venture to correct a probable misprint—apparently also saccharose even after incubation for seven days.

It has to be emphasized, however that numerous other workers, when examining freshly isolated strains, never met such aberrant reactions At the same time it was established, however that a considerable number of cholera like vibrios including those isolated from surface waters, also gave the Group I reactions Thus Pollitzer (1936) found that practically one third (32) of 100 Shanghai water vibrios belonged to this group Taylor Read & Pandit (1936) comparing the reactions of 125 cholera strains with those of 369 cholera like strains isolated from patients showing clinical signs of the disease, from carriers and from surface waters, found that 26 8% of the cholera like vibrios from human sources as well as 11 4% of the water vibrios showed the reactions of Heiberg s Group I and concluded " that fermentation tests with these three sugars will not provide accurate information as to the characteristics of the vibrios which can be obtained by serological tests " However as noted below in a subsequent paper (1937) Taylor and his co-workers ascribed some usefulness to Heiberg s method if used in combination with other biochemical tests Possibly also as claimed by Heiberg, the method will prove of value for the classification of the cholera-like vibrios which are rather heterogeneous serologically

Voges-Proskauer reaction

In the course of a study on the bacteria of the haemorrhagic septicaemia group Voges & Proskauer (1898) found that a red colour was produced if a few drops of a strong solution of potassium hydrate were added to growths of these organisms in glucose-containing media. As was afterwards established, the reaction depends upon the formation of acetylmethylcarbinol in the course of glucose decomposition, which has been ascribed to the action of a special enzyme, " carboligase " (see O Meara, 1931)

Lemoigne (1920) using a rather delicate reaction (nickel-dimethylglyoxine test) for the examination of culture distillates, found that small quantities of acetylmethylcarbinol were formed by both cholera and cholera like vibrios. However while his method thus appeared to have no differential diagnostic value, it was in Lemoigne s opinion potentially useful for the characterization of different races of these organisms.

Attention to the possibility of using the Voges-Proskauer reaction for the examination of cholera and cholera like vibrios was, as far as could be established, first drawn by Taylor in a report to the Scientific Advisory Board of the Indian Research Fund Association rendered in 1936 and quoted by Baars (1938) Reporting in detail on these investigations in a valuable *Study of the vibrio group and its relation to cholera*, Taylor Pandit & Read (1937) stated that they had compared the modified Voges-Proskauer

reaction according to Barritt (1936) with the original method, using the following technique

(a) For Barritt's modified test the glucose phosphate medium recommended by the Ministry of Health (Report No 71 1934) with an initial pH of 7.5 was distributed in 6 × 5/8 tubes. These were inoculated rather heavily and incubated at 37°C for 3 days. About one ml of the culture was then transferred to a tube and to it was added, first, 0 6 ml of a 5 alcoholic solution of α-naphthol and then 0 2 ml of a 40 % KOH solution. Results were read at the end of 4 hours A positive result was indicated by the appearance of a pink colour on the surface of the fluid in about 5-10 minutes already which then deepened and spread to the bottom of the tube In negative cases the fluid usually remained colourless but sometimes a faint brownish tinge appeared.

(b) To carry out the original test, 40 KOH solution was added to the culture tubes in amounts of 0.25 ml after the transfers necessary for the α-naphthol tests had been made. Results were read after 4 and once more after 24 hours.

Carrying out these tests with 90 classical cholera strains 6 El Tor strains and 351 cholera like strains Taylor Pandit & Read obtained in many instances a positive result with the aid of Barritt s modified technique only The reverse i e a positive result with the original Voges Proskauer technique and a negative one with Barritt s modification was never observed

Combining the observations they made with the aid of Barritt s test, the cholera red reaction and sugar fermentation tests according to Heiberg, Taylor and colleagues obtained the following important results

(1) *Classical* V cholerae All non haemolytic strains agglutinable with cholera immune serum belonging to Heiberg s Group I gave a cholera red reaction but were *negative* to the modified Voges Proskauer test.

(2) *El Tor strains* Out of the six haemolytic strains which were agglutinable with cholera immune serum five differed from the classical type in so far as they gave a *positive* modified Voges Proskauer reaction

(3) *Cholera-like vibrios* The vibrios inagglutinable with cholera immune serum were found to fall into two main groups

(a) A larger group (240 strains) showing both a positive cholera red and modified Voges Proskauer reaction and consisting mainly of strains of Heiberg Groups I and II

(b) A minority cholera red and Voges-Proskauer negative, belonging with few exceptions to Heiberg Groups III VI while one strain was found to fall into a hitherto unknown fermentation group (saccharose negative arabinose and mannose positive)

Commenting on these observations Taylor Pandit & Read stated the following

* Mention has already been made that agglutinable non haemolytic vibrios tested gave the reaction C R (cholera-red) + V P (Voges-Proskauer)— In the series of 351 inagglutinable strains examined, only 15 gave the same results and of these 10 were of types aberrant in their sugar reactions from the recognized Heiberg types. No inagglutinable strain of

Heiberg type I has given the reactions C R + V P— It is therefore possible, on biochemical evidence alone, to obtain presumptive diagnosis of the serology of the typical *V cholerae* If it gives fermentation reactions of Heiberg type I, is cholera red positive and negative to the modified V-P test, it is very probably an agglutinable vibrio "

Taylor Pandit & Read claimed in this connexion that, if rather large inocula were used, it was permissible to carry out Barritt s test after an incubation of only one day instead of the customary three days and that consequently the fermentation, cholera red and modified Voges-Proskauer tests could be " profitably performed, along with the agglutination test and read with it " It has to be pointed out, however that, using the now available sera, great reliance can be placed upon slide agglutination tests made as soon as suspicious colonies are found on the plates used for primary isolation of *V cholerae*

The important observation that, in contrast to most classical *V cholerae* strains the El Tor vibrios usually give a positive Voges-Proskauer reaction, was confirmed by several workers such as de Moor (1938, 1949) *Mochtar & Baars (1938) Gispen (1939) Marras (1940) and Paris & Gallut* (1951) Baars (1940) maintained in this connexion that the El Tor vibrios were capable of forming acetylmethylcarbinol only under aerobic but not under anaerobic conditions Gallut (1946) found, like Lemoigne in 1920 that, if tests more sensitive than the Voges-Proskauer reaction were used the cholera vibrios could be also proved to produce this substance However, the El Tor vibrios acted far more energetically in this respect This is in accord with the observation of Baars (1940) that these vibrios ferment sugars far more energetically than the *V cholerae* under aerobic conditions and to some extent even under anaerobic conditions

Haemodigestive and haemolytic properties

Observations on the reactions produced by *V cholerae* in blood-containing media go back to a rather casual statement made in 1884 by Koch at a cholera conference in Berlin (*Berliner klinische Wochenschrift*, 1884) to the effect that in one instance when blood-containing stools had been used to make a gelatin plate clear zones became visible round the cholera colonies. Koch felt entitled to conclude from this observation that *V cholerae* was capable of destroying erythrocytes and probably also other cells Schottmüller (1904) also ascribed haemolytic properties to the cholera vibrios which facilitated the differentiation of these organisms from other intestinal bacteria.

The observations of Bitter (1886) on the action of the "ferment" of *V cholerae* on rabbit blood suspensions cannot be considered conclusive because he worked with culture fluids which had been heated for half an hour at 60°C. He found that under these circumstances the erythrocytes were remarkably resistant to the action of the ferment. The haemolytic action of *V cholerae* on blood-containing gelatin plates, ascribed by other

workers to the secretion of a cell-destroying toxin by the organisms was in Bitter's opinion due to the damage caused to the erythrocytes through enclosure in the media, on account of which the blood corpuscles became amenable to the action of the ferment and other products of decomposition

An unequivocal claim that *V cholerae* like some other micro-organisms produced a haemolytic enzyme was made by Eijkman (1901) but there can be no doubt that the halo formation on blood-agar plates described by him was the result of haemodigestion (see below) and not of true haemolysis

Studying 12 cholera like as well as 9 true cholera strains, Kraus (1903) found the former alone capable of producing a soluble "haemotoxin" in broth cultures and consequently able to produce zones of clearing round their colonies on blood-agar plates Kraus recommended therefore, the use of the latter media for the differentiation of the non haemolytic *V cholerae* from haemolytic cholera like organisms

The problem of the haemolytic properties of the vibrios began to attract much attention after Gotschlich (1905 1906) had isolated six peculiar strains from dead bodies of returned Mecca pilgrims at the quarantine camp of El Tor Though these victims showed no signs of choleraic disease either during life or *post mortem*, the vibrios found in their intestines were not only agglutinable with cholera immune serum but showed as far as the tests used by Gotschlich went, in all other respects as well, the reactions of true cholera vibrios However, re-examining these strains, Kraus & Přibram (1905) found to their surprise that the organisms in question produced, like the cholera like vibrios formerly examined by Kraus a soluble haemotoxin as well as an exotoxin rapidly lethal to experimental animals

Since this discovery was made, diametrically opposite views have been expressed in regard to the relationship between these El Tor vibrios[1] with the true cholera vibrios responsible for epidemics and—in connexion with this problem—regarding the question whether or not the classical *V cholerae* is non-haemolytic in contrast to the El Tor vibrios. Kraus and his co-workers (see the ultimate statement of Kraus, 1922) continued to assert that on account of its above-described properties the El Tor vibrios fell into a class distinct from that of the non-haemolytic *V cholerae* Many German workers on the contrary (see summary by Kolle & Prigge, 1928) maintained that the cholera vibrios were apt to show variability in regard to their haemolytic properties as well as in other respects and that consequently tests with blood-containing media were unsuitable for the characterization of this organism—an opinion which implies that the El Tor vibrios do not form a group of their own

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[1] Though a few workers have designated also haemolytic cholera-like vibrios with this name, it is imperative to use it exclusively for those haemolytic strains which are agglutinable with cholera-immune serum. Otherwise utter confusion would reign.

In order properly to assess the merits of these opposite claims, it is necessary to pay attention to the methods of examination used by the various workers and to the manner in which they interpreted their findings.

Proper choice of blood

The first point to be noted in this connexion is that the various workers have used different sorts of erythrocytes for their tests As noted above, Koch (1884) made his initial observation on the supposed haemolytic properties of *V cholerae* on a plate which happened to contain human blood. The use of this was recommended by Schottmüller (1904) while some other early workers (e.g. initially Kraus, 1903) used rabbit blood for their tests. Prausnitz (1905) who seems to have been the first to make comparative tests in this respect, found rabbit as well as calf blood more suitable than human blood but worked for the sake of economy mainly with calf blood. The use of the latter was strongly recommended by Schumacher (1906) because in his experience the calf erythrocytes were the least liable to become damaged by mechanical, thermic, or chemical influences and were, therefore the most resistant to the action of the vibrio "ferments" Kraus and his co-workers on the other hand (see Kraus & Prantschoff 1906) soon adopted the use of sheep blood but considered goat blood also suitable Goat blood has been used for the large-scale studies on the haemolytic properties of *V cholerae* referred to below but, as confirmed by some later observers, for instance on account of comparative tests by Finkelstein (1930), sheep blood was equally satisfactory In fact Krishnan & Gupta (1949) submitting in 1949 to the WHO Expert Committee on Cholera a draft proposal for a standard haemolytic test to be adopted for cholera work, recommended the use of sheep blood in preference to that of goat blood.

These statements make it clear that in assessing the results of past workers, full reliance can be placed only on findings with suitable types of blood, particularly with goat sheep, or calf blood, while those with human blood ought to be disregarded It is of great interest to add that according to observations made by Zimmermann (1934) most cholera strains, though incapable of producing lysis of sheep erythrocytes were found able to form a thermolabile haemolysin against human red blood corpuscles, while the El Tor vibrios lysed both sorts of blood. These observations which have been recently confirmed by De and co-authors (1954) are in accordance with earlier findings made by Přibram & Russ (quoted by Kolle & Schürmann, 1912 and Kolle & Prigge, 1928) who carrying out absorption tests, showed that the filtrates of vibrio cultures did not contain one common haemolysin but separate ones for the different sorts of erythrocytes they were able to lyse

Methods of examination

Two fundamentally different methods are used to assess the behaviour of cholera or other vibrios in blood-containing media—cultivation of the organisms on blood plates (nowadays invariably agar plates) and tests with blood suspensions which have been added to adequate amounts of fluid vibrio cultures, or of their filtrates or centrifugates The technique usually adopted for the latter purpose which, as will be set forth in a later chapter is still used in actual practice with some modification, is well exemplified by the following description of the classical procedure adopted by Greig (1914b)

"Each strain was grown in alkaline broth, as recommended by Meinicke (1905) for 3 days at 37°C., at the end of that period falling quantities of the culture, viz., 1 c.c., 0.5 c.c., 0 1 c.c., 0.05 c.c. and 0.01 c.c. were measured with a pipette and placed in small sterile test-tubes the quantities were brought up to exactly 1 c.c. in each tube with 0 85 % NaCl Then 1 c.c. of a 5 % suspension of goat s washed red corpuscles was added to each tube. An experimental error is made if the suspension of red corpuscles is added first, since the culture, which is lighter floats on the top so that if a haemotoxine is present the upper layer of red corpuscles gets a very concentrated dose. The contents of the tube are very carefully mixed and the mixture is placed in the incubator at 37°C. for 2 hours. The tubes are taken out and placed in the icechest over night. Next day the presence or absence of haemolysis in each tube is noted and recorded"

No doubt, it would be more exact to use corresponding amounts of filtrates instead of materials from the fluid cultures for the above-described tests. Unfortunately however as first shown by Meinicke (1905) and con firmed by later observers the haemolytic property of the strains is greatly reduced if filtration is resorted to Greig (1914b) maintained in this respect that "the haemolysis producing substance in the broth culture is to a considerable extent non filterable"

It will be perceived that, whereas in the case of tests performed accord ing to Greig s or a similar technique the red blood corpuscles are exposed almost solely to the action of the "haemotoxins" (haemolysins) in the case of cultivation on solid blood-containing media, they are also exposed to the action of the enzymes of the vibrios It is not surprising, therefore that, as will be shown below, the results obtained with these two categories of tests respectively are as markedly different as the technique adopted in each case It is obvious that the results of tests aiming to show the presence or absence of haemolysis will be far more clear-cut if, by using Greig s or a similar technique, or by working with filtrates, the additional influence of the vibrio enzymes is practically or totally excluded

Quality of the media used

As noted by Schumacher (1906) in the course of exhaustive studies on the behaviour of cholera and cholera like vibrios on blood-agar plates it is essential to pour these with a sufficient amount of the medium so as

to obtain a uniformly and adequately thick layer The reason for this was that even vibrios, which ordinarily did not produce zones on the plates, were apt to show ill-defined haloes round their colonies at thin spots of improperly poured plates. Loewy (1915), besides repeating the advice given by Schumacher also insisted upon the use of *freshly* taken and defibrinated blood, because blood kept in storage could show spontaneous haemolysis. Plates which had become dry or which showed a darkening of their initially bright red colour were unsuitable for haemolysis tests

Zimmermann (1932) noted, in analogy with the experiences of Meinicke (1905) in the case of blood plates, that the results of haemolysin tests made by growing cholera vibrios for 48 hours in broth tubes to which sheep blood had been added previously to obtain a concentration of 5%, were apt to be divergent if the tests were repeated at short intervals It was striking, however that different strains tested at one and the same time showed a peculiarly uniform behaviour either mostly producing haemolysis or mostly failing to do so Since such a simultaneously occurring variation of several strains was altogether unlikely, Zimmermann postulated with much reason that the observed variations in the haemolytic properties of the strains were the result of differences in the physicochemical state of the media or of corresponding changes taking place in the course of cultivation. Inadequacies in the defibrination of the blood were likewise apt to introduce an element of chance. In fact, consistently negative results were obtained with the same strains if instead of the broth a synthetic fluid medium and, instead of defibrinated blood, citrated sterile sheep blood were used. However, significant though these findings are in actual practice it is equally reliable and more expedient to use an up-to-date modification of Greig's method in place of that of Zimmermann It seems unnecessary therefore, to deal in detail with the technique of the last mentioned worker

Status of the strains examined

The various workers postulating an inconstancy of the reactions produced by vibrios in blood-containing media or suspensions based their claims, to a varying extent, upon an examination of recently isolated growths and of stock cultures respectively It is of importance, therefore, to see whether or to what extent, the inconstancies which they noted in the course of their work were due to a changing reactivity of the individual strains, caused by the process of ageing and/or by mutation or dissociation

Studying the mutations of *V cholerae* Baerthlein (1911b, 1912, 1918) noted that the opaque variants of ordinarily non haemolytic cholera vibrios were able to produce haemolysis in blood suspensions as well as clear zones round their colonies on blood-agar plates. Since however this worker continued to keep both the suspensions and the plates at 37°C

in the incubator and extended the period of observation to 72 hours no reliance can be placed on his findings Further observations made by Goyle & Gupta (1932) with spontaneously agglutinating cholera strains which had obviously undergone dissociation and by Genevray (1940) with variants of *V cholerae* obtained through the action of chlorine or phenol showed that like their smooth parent-strains these dissociants and variants respectively failed to produce haemolysis in blood suspensions

Van Loghem (1913b) and Snapper (1921) asserted in general that, while the haemodigestive properties of *V cholerae* were apt to show variation the incapability of these organisms of producing haemolysis in fluid substrates, as well as the haemolytic properties of the El Tor vibrios were stable characteristics This is in accord with previous observations made by Meinicke (1905) who stated that

" the haemolysins of the vibrios are but little apt to undergo spontaneous decomposition. Out of 20 filtrates of different vibrios cultures, to which phenol had been added and which had then been kept in the ice-box for 6 months 17 retained their original titre and three only had become less haemolytic. " [Trans.]

Analogous observations were made by Zimmermann (1933) who found that, with the exception of one variable El Tor strain none of the vibrio strains studied by him showed evidence of a short term variation of their haemolytic properties Re-examining these strains once more after a period of observation totalling one year and nine months Zimmermann (1934) likewise observed no instance of a fundamental change in their haemolytic properties and only in a limited number of instances a variation in the intensity of the reactions produced by haemolytic strains The haemolytic properties of Zimmermann s strains were not influenced by animal passage, nor by bacteriophage action as had been claimed by Doorenbos (1932)

Though, as will be gathered from the evidence set forth above, the presence or absence of haemolysis may be considered stable characteristics of practically all strains of the cholera and allied vibrios [1] it is nevertheless desirable to use freshly isolated growths rather than stock cultures to assess the reactions produced in this respect by a given strain or series of strains Van Loghem (quoted by Zimmermann 1932) no doubt went rather far when ascribing the occurrence of aberrant haemolytic reactions shown by stock cultures of *V cholerae* to contaminations with haemolytic vibrios But even apart from this possibility the uncertainties arising from the use of stock cultures the source of origin and character of which are quite often not or not exactly known may be great, and this absence of exact information was no doubt responsible to quite a considerable extent for the statements made to the effect that the classical *V cholerae* may produce true haemolysis The difficulties apt to arise in

[1] The only recent observations recorded to the contrary were those of Del Favero (1938) who stated that laboratory strains of *V cholerae* subjected 15 times to subcultivation at 20°C, became, in contrast to their initial behaviour strongly haemolytic for sheep-erythrocytes. Since Del Favero s original paper could not be consulted, details of his methods could not be ascertained.

this respect are well exemplified by the experiences of Zimmermann (1932) This worker found among the 70 strains labelled in his material as *V cholerae* two which were not agglutinable with cholera immune serum but were haemolytic, and which, therefore as he cautiously put it could not be considered "typical" cholera vibrios Out of Zimmermann s 21 strains labelled as El Tor, on the other hand, one proved to be non haemolytic, thus reacting like a cholera vibrio and not like an El Tor vibrio A minority of his other El Tor strains were but slightly or even almost not agglutinable with cholera immune serum, but—as Zimmermann argued—"they must have been found agglutinable with cholera serum by Gotschlich and Doorenbos, because they had been diagnosed on account of this fact"

Interpretation of results

The fundamental difference between the phenomenon of true haemolysis observable in fluid substrates and the appearances apt to become manifest when the vibrios were grown on blood plates was clearly recognized by Schumacher (1906) who maintained in this respect that

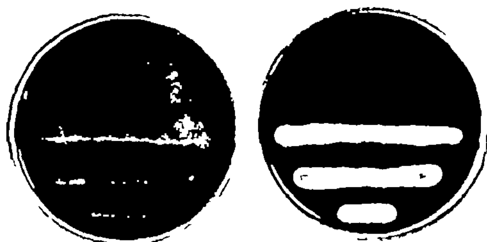
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However even though Schumacher and also some other early workers emphasized that, in order to decide whether or not a given strain was haemolytic, tests should be made with blood suspensions and not with blood plates, many investigators not only mainly paid attention to the latter category of tests but often took the appearance of clear zones round the vibrio colonies on the blood plates as proof that the strains in question were endowed with haemolytic properties.

It was the great merit of Van Loghem (1911 1913a, 1913b) to have reaffirmed through studies commenced in about 1909 that the classical cholera and the El Tor vibrios respectively produced qualitatively distinct reactions in blood-agar plates besides being distinguishable by their behaviour in blood-containing fluid substrates, in which in contrast to *V cholerae* the El Tors produced haemolysis (Fig 19) It is true that *V cholerae* was capable of producing clear zones round its colonies on goat blood agar plates as the El Tor vibrios invariably did However Van Loghem emphasized, in the case of the latter organisms, that these zones appeared quickly were *not* quite transparent, and showed a reddish tint. In the case of *V cholerae* on the contrary the zones appeared more slowly were quite clear and had a greenish hue Spectroscopically it could be shown that oxyhaemoglobin, while absent in the zones around the cholera colonies, was present in the zones surrounding the El Tor colonies, because in their case true *haemolysis* took place which led to the penetration of haemoglobin into the zones. The

FIG 18. HAEMOLYSIS AND HAEMODIGESTION OF 48-HOUR-OLD VIBRIO CULTURES IN PETRI DISHES ON NUTRIENT AGAR WITH 5 % SHEEP RED CELLS

Streak seeding



Left : Haemolytic El Tor vibrio, non-haemodigestive

Right : Haemodigestive *Vibrio cholerae* non-haemolytic

Spot seeding



Left : Haemolytic El Tor vibrio non-haemodigestive

Right : Haemodigestive *Vibrio cholerae* non-haemolytic

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lection produced marked haemolysis and the same was true of the 14 cholera like strains at his disposal Of the two haemolytic cholera strains one was a stock strain over 20 years old which agglu inated but weakly with cholera immune serum, the other a stock strain from Paris kept in the Berlin Institute of Hygiene

Genevray & Bruneau (1938c) recording their observations on more than 500 Indochinese cholera strains stated that they had observed no instance of haemolysis produced by these vibrios within 24 hours However, all the strains produced *hématolyse* (that is apparently haemodigestion) on sheep blood as well as on rabbit blood agar

Attention has also to be drawn to the experiments made by a few workers with heated blood agar plates ("chocolate" agar plates) on which, as was first noted by Loewy (1915) cholera vibrios were apt to produce clear zones. Kovacs (1927) exhaustively studying this phenomenon confirmed that these vibrios obviously because they were capable of exerting an action on the erythrocytes which were damaged through boiling, produced—usually on the second day of incubation—yellowish, quite transparent haloes round their colonies on chocolate agar (*Kochblutagar*) plates prepared with the aid of sheep blood El Tor vibrios on the contrary because they exerted at most a slight haemodigestive action produced but occasionally indistinct haloes

Finkelstein (1930) postulated that by the combined use of blood suspensions, ordinary and heated blood agar the vibrios could be classified into four groups thus

Group	*Haemolysis in suspensions*	*Clearing of blood-agar*	*Clearing of heated blood-agar*
I	negative	negative	negative
II	positive	positive	positive
III	positive	positive	negative
IV	negative	positive	positive

In contrast with the findings of Loewy and Kovacs, Finkelstein claimed that the classical *V cholerae* does not produce clearing of heated blood agar plates, thus falling into Group I of his scheme. Since, however he used ox blood for his tests and took readings after 24 hours incubation at 37 C only and since, moreover a study of his protocols shows that out of the total of 11 strains isolated from cases of clinical cholera which he could examine *5 only fell into Group I* while 3 belonged to Group II 2 to Group III and 1 to Group IV it is impossible to place reliance in Finkelstein s findings Generally speaking, it must be emphasized once more that tests on blood plates, however interesting their results may be are of no decisive value in answering the practically most important question of whether or not a given vibrio strain is capable of producing true haemolysis Tests with blood suspensions alone can furnish clear-cut evidence in this respect.

found to be capable of bringing about in successive stages "the phenomenon of haemodigestion" as described by Van Loghem, and (*b*) was also found to exert a tryptic action both on denatured proteins such as coagulated horse serum gelatin, and milk, and natural proteins such as egg-white and fibrin. The results of these investigations as well as those of the extraction of an exohaemolysin from culture media used for growing El Tor vibrios which were recorded by Bernard Guillerm & Gallut in 1939 and will receive full attention in the next chapter definitely confirm the difference of the reactions produced by cholera and El Tor vibrios in blood-containing media or substrates, thus ending a controversy lasting for more than thirty years

The experiences of Greig (1914b) and other modern workers in testing representative series of vibrio strains almost invariably[1] supported the validity of the above mentioned observations

Greig (1914b) found that the 333 cholera strains which he examined according to the above mentioned technique all proved non haemolytic, while 100 strains of cholera like vibrios isolated from human stools or from surface waters, invariably produced haemolysis, some to a marked degree A great majority of the 161 cholera strains the behaviour of which was tested on agar plates containing 12% goat blood, produced no definite zones of clearing in these media within 24 hours and only a few indistinct ones. However clear zones became manifest, if readings were taken after more prolonged incubation Greig emphasized therefore, that, if blood plates were *used to test the haemolytic properties of cholera-suspect vibrios*, positive findings becoming manifest after more than 24 hours should be disregarded.

Testing 103 cholera strains which included, besides 27 stock cultures, mainly those isolated in Romania and Bulgaria, Loewy (1915) found no evidence of haemolysin production either in the centrifugate of 5 days broth cultures or in the case of agar plates to which 10% sheep or goat blood had been added However a proteolytic "ferment" of the *V cholerae* which in Loewy s opinion was identical with the ferment causing gelatin liquefaction, was found by this worker to be capable of exerting a digestive action on damaged erythrocytes.

Van Loghem, summarizing in 1932 the results of the above-mentioned observers as well as those obtained by various workers in the Dutch East Indies, was able to report on the examination of over 600 strains isolated from authentic cholera cases and invariably found to be non-haemolytic.

As Zimmermann summarized in 1933 among the 69 cholera strains examined by him two were found to possess haemolytic properties. With one exception, which has been noted above the 28 El Tor strains of his col-

[1] A divergent opinion was expressed by Kabeshima who, in short note published in 1918, maintained that 91.6% of the 206 cholera strains investigated by him showed haemolysis in fluid as well as on solid media. It was not possible to consult the more exhaustive publication in Japanese medical journal which Kabeshima referred to in his note. There can be hardly any doubt, however that deviations from the standard technique used by the other workers were responsible for the strikingly aberrant results obtained by him.

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[1] A divergent opinion was expressed by Kabeshima who, in short note published in 1918, maintained that 91.6% of the 206 cholera strains investigated by him showed haemolyses in fluid as well as on solid media. It was not possible to consult the more exhaustive publication in Japanese medical journal which Kabeshima referred to in his note. There can be hardly any doubt, however that deviations from the standard technique used by the other workers were responsible for the strikingly aberrant results obtained by him.

Recent noteworthy observations on the occurrence of El Tor vibrios in India may be summarized as follows Venkatraman and co-authors (1941) recorded that they isolated 15 El Tor strains from 878 specimens collected in the Tanjore district of Madras from 237 open natural water sources including rivers channels tanks ponds and a few wells The examination of 1827 stool samples gave negative results for El Tor vibrios and since cholera was absent at the time also for *V cholerae*

Read & Pandit (1941) carried out analogous investigations in (*a*) two districts of Bengal where cholera was endemic (*b*) a district in Bihar where annual epidemics occurred and (*c*) an area in Sind which had remained largely free from cholera for the past ten years The main conclusions reached by these two workers were

"[1] The non haemolytic agglutinable vibrio was found in all except one of the clinical cases in areas where the presence of cholera could be established, provided the examination was carried out sufficiently early in the disease

"[2] About 7 per cent of close contacts of cholera cases proved positive and about 16 per cent of water sources in direct contact with cases were positive at different periods of the epidemic On the other hand the non haemolytic vibrio with one or possibly two exceptions was not found (in water samples) in the absence of the disease.

"[3] The haemolytic agglutinable vibrio while detected in the presence of the disease, has been found usually in its absence It has been found in cholera areas of two different epidemiological types in different provinces of India and in relative large numbers (i.e. in 18 out of 206 water samples) in an area which must be taken as not only free from cholera during the period of investigation but free from cholera during the decade previous."

Continuing these investigations Read Pandit & Das (1942) tested the haemolytic properties of cholera and El Tor strains from various sources Growing these organisms in Douglas s broth (see Douglas 1914) instead of in untrypsinized broth as had been done by Greig but otherwise using the technique recommended by the latter worker Read Pandit & Das obtained the following results

Character of strains	*Haemolysis* produced	*Haemolysis* not produced
Case strains, India	0	15
Case strains, Celebes	7	0
Contacts India	0	4
Contacts, Celebes	3	0
Water India	15	6
Water Celebes	6	0
El Tor stock strains	5	1
Total	36	26

* All agglutinable with cholera-immune serum.

It will be noted that while the strains isolated from cholera patients and contacts in India, in contrast to those from Celebes, were invariably non haemolytic, the majority of the strains isolated from water sources in India

In addition to the above recorded findings, within recent years the following important observations have been made in regard to the El Tor vibrio in particular

As has been noted in Chapter 2,[1] in 1937 38 as well as in 1939-40 and in 1944 manifestations of a "choleriform" disease with a high fatality rate have been observed in South Celebes in which haemolytic vibrios agglutinable with cholera immune serum were found to play the causative role (de Moor 1938 1949) Making a careful study of 370 strains isolated from sufferers and their environment, de Moor (1949) concluded that the vibrios in question which fell in the same serological group as *V cholerae* and belonged like the latter to Group I of Heiberg, but gave with four exceptions a positive Voges Proskauer reaction were true El Tor vibrios Agreeing with Van Loghem (1938) that the El Tor vibrio fell into a group different from that of *V cholerae* de Moor proposed the name "Para cholera (El Tor)" for the choleraic disease in Celebes. It is thus curious to see this term once more used in the sense proposed by Kraus (1909) i e. to designate instances of choleraic disease caused by haemolytic vibrios agglutinable with cholera immune serum Since, however the name "para cholera" was afterwards adopted to designate clinical manifestations in which vibrios serologically different from *V cholerae* were assumed to have played a causative role it cannot be considered as felicitous. The term "enteritis choleriformis El Tor" proposed by Van Loghem (1938) to designate the Celebes disease seems therefore preferable.

Though the outbreaks observed in Celebes have been the most conspicuous, they were probably not the first in which El Tor vibrios were responsible for the causation of choleraic disease. A strain called Kadiköj which showed the properties characteristic of the El Tor vibrios, was isolated in 1913 by Kraus in Bulgaria (Kovacs 1927) while Hoppe Seyler (1916) claimed that cholera vibrios with haemolytic properties had been responsible for a limited outbreak in Kiel and added that *V cholerae* strains of this kind had been met with repeatedly in Poland during the First World War Mackie (1929) maintained in general that vibrios of this type had been met in choleraic conditions as well as in carriers in countries outside India, e g. in the Near East.

It is of great interest to note in the latter connexion that Abdoelrachman (1944-45) examining 90 water samples from different sources in the Hejaz, was able to demonstrate the presence of El Tor vibrios in one out of 29 specimens taken from the holy well Zam-Zam at Mecca. All other water samples examined by this worker as well as 1109 stool samples, including those of 715 pilgrims from the Dutch East Indies and Malaya, gave negative results for the El Tor vibrio and cholera being absent at the time also for *V cholerae*

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[1] See page 64.

In the experience of Borntraeger (1892) dry heat of 80°C killed the cholera vibrios within a few minutes while exposure to higher degrees of dry heat (80° 100°C) led to the death of the organisms within a few seconds Borntraeger considered it feasible under these circumstances to use, in emergencies, dry heat generated in baking stoves for the dis infection of objects such as clothing and bedding contaminated with *V cholerae*

It is of interest to add that Shousha (1924) found the rough dissociants of *V cholerae* somewhat more resistant to heat than the smooth parent strains

Cold

Though not very resistant to heat the cholera vibrios show a remarkable tolerance for low temperatures, even for those well below the freezing point [1] Uffelmann (1893a) established in this connexion that suspensions of *V cholerae* in water as well as cholera cultures if exposed in the open during winter remained viable for 3–4 days even when the minimal temperature became as low as −24 8°C. Still more remarkable experiments carried out by Kasansky (1895) showed that broth, gelatin and agar cultures of *V cholerae* (*a*) tolerated temperatures down to −31 8°C (*b*) remained viable if kept completely frozen for 20 days or if subjected to repeated freezing and thawing, and (*c*) survived exposure to the cold of the winter at Kasan, Russia, for four months However cultures exposed to the cold in November proved to be no longer viable when re-examined in April or May The survival of cholera vibrios in artificially contaminated raw river water samples under the conditions prevailing in winter (December to mid May) in Berlin for a maximum period of a little over four months has been recorded by Christian (1908)

Drying

As Koch recorded at a cholera conference held in Berlin in 1884 (*Berliner klinische Wochenschrift* 1884) cholera vibrios, grown in peptone broth and spread in thin layers on cover glasses withstood drying for periods of up to one hour but were sometimes found to have succumbed after two hours and were invariably incapable of surviving drying for periods exceeding three hours If compact masses of vibrios, scrapings from potato-cultures for instance were dried, the organisms could survive for periods of up to 24 hours, evidently because under these circumstances no rapid drying took place

Similar experiments were made by Kitasato (1889a) who found that on silk threads which had been dipped into fluid cultures the cholera

[1] Summaries of the early observations made in this respect have been furnished by Dunbar (1896) and by Christian (1908)

showed the haemolytic properties characteristic of the El Tor vibrios This was particularly true of the growths obtained from " non-contact " water sources 14 out of 15 of which proved haemolytic

In marked contrast to these observations Mukherji (1955) claimed that El Tor type vibrios had been responsible for a cholera outbreak at Lucknow in 1945 However since (*a*) bacteriological examinations seem to have been made in but one half of the patients showing clinical signs of the infection and (*b*) only 13 of the 25 strains found to be agglutinated by cholera immune serum proved positive in Greig tests Mukherji s claim does not seem con vincing. It is quite possible that, just as cholera-like vibrios of obviously aquatic origin are quite frequently found side by side with or in place of *V cholerae* in the stools of patients showing typical signs of cholera during epidemics so the presence of the haemolytic vibrios noted by Mukherji was of an accidental nature being due to the occurrence of this type of organism in the environment of the sufferers Nevertheless, in view of the observations made in Celebes it would be unwise to deny the possibility that El Tor type vibrios can be responsible for occasional choleraic mani festations in man At the same time however it would seem unjustified thus far to doubt that, as Gardner & Venkatraman put it,

" the absence of haemolytic power and the possession of a characteristic O antigen are the chief distinctive characters of the vibrios most undoubtedly causative in epidemic cholera "

Vital Resistance

Heat

It is generally agreed that *V cholerae* is not at all resistant to high temperatures As Kolle & Schürmann (1912) summarized in this connexion

" Boiling temperature destroys the vibrios immediately At 80°C they are killed with certainty within five minutes, and heating for half an hour at 56°C suffices to terminate the life of the cholera vibrios. [Trans.]

Babes (1885) established that rapid heating of gelatin cultures to even only 75 C rendered the growths sterile Good growths could be obtained from gelatin cultures slowly heated (? in the water bath) up to 45°C. Ex posure at 46 -48°C for two days rendered the cultures sterile but tem peratures of 40 -41°C were well tolerated by the cholera cultures for three days

Kitasato (1889a) heating gelatin tubes which after liquefaction had been inoculated with *V cholerae* at various temperatures and for different lengths of time in the water bath and then making roll cultures, found that (*a*) exposure of the inoculated tubes for 15 minutes to 55 C usually pre- vented growth, and (*b*) heating for 10 minutes at 60°C or for 5 minutes at 65°C invariably did so

Sunlight

Orsi (1907) carrying out systematic studies with cultures of *V cholerae* and *Salmonella typhosa* found that sunlight exerted a damaging action on these organisms without however, invariably leading to their total destruction The cholera vibrios in particular remained viable in fairly considerable numbers after exposures to sunlight averaging 8-10 hours (temperature in the shade 23° 31°C) In the experience of Conor (1912) however, these organisms, if suspended in canal water and exposed to the action of sunlight in transparent glass flasks or tubes were no longer demonstrable after an exposure of 5-7 hours at temperatures ranging from 27°C to 34 6°C. Yasukawa (1933) working with suspensions of *V cholerae* in sterilized sea water even found that a two hours exposure of these specimens to sunlight in boxes covered with transparent or with cobalt glass was sufficient to kill the vibrios

Other rays

Schiavone & Trerotoli (1913 see also Galeotti, 1916) found that cholera vibrios if suspended in saline and exposed in glass dishes in thin layers (2 3 mm) to the rays of a mercury vapour lamp 20 cm distant, were killed in one minute even though the temperature did not exceed 20°C. Under the same conditions the vibrios in blood serum were killed in half an hour those in broth or urine in 2 hours, and those in milk in 2½ hours Pieces of material soaked with suspensions of *V cholerae* were rendered sterile by the ultraviolet rays of the lamp in 15 to 45 minutes

As shown by Rieder (1898) exposure of cholera vibrios to X rays for 20-30 minutes is apt to inhibit their growth or even to kill the organisms.

Action of supersonic waves (Fig. 20)

As established by Violle (1950) the action of supersonic waves on saline suspensions of *V cholerae* led as a rule to complete lysis of the organisms Since however supersonic irradiation was capable of killing the vibrios without visibly changing them stained preparations of treated suspensions were apt to show all transitional stages from typical organisms to complete disappearance A further interesting observation was that as shown by tests with methyl red the irradiation led to a change of the pH of the vibrio suspensions, the yellow-orange colour of the suspensions changing after treatment for about ten minutes to red and then to pink, and finally quite disappearing

Acids

As alluded to earlier in this chapter, *V cholerae* is extremely sensitive to the action of acids In this connexion Kolle & Schürmann (1912) stated

vibrios withstood drying better than on cover-glasses, obviously because desiccation took place more slowly Working at room temperature (20 22°C) Kitasato found that even on the silk threads the vibrios survived for a few days only longest (up to seven days) on those kept in the exsiccator apparently because in the latter case rapid drying of the outer layers led to a more prolonged retention of some moisture inside the threads.

However in an additional note Kitasato (1890) stated that if kept on moist filter paper in closed Petri dishes cholera vibrios on cover-glasses were capable of surviving for 85-100 days, and for 200 days or even longer on silk threads

Suzuki (1922) again studying the resistance of *V cholerae* to drying, found that if the organisms were smeared on a silk thread which was then placed in a jar with calcium chloride, the organisms survived no longer than 18-28 hours However longer survival took place if suspensions of the vibrios in saline solutions containing some horse serum, egg-white, or undiluted horse serum were used for such tests.

Observations made by some workers have shown that when undergoing exsiccation cholera vibrios from layers which apparently had become quite dry may remain subcultivable Thus Gildemeister & Baerthlein (1915) studying the survival of *V cholerae* in the faeces of patients and carriers (see below) sometimes obtained positive results when making subcultures from apparently exsiccated specimens

Laigret & Auburtin (1938) recorded that they had obtained broth subcultures from cholera vibrios which had been dried *in vacuo* over calcium chloride ground in a mortar and then kept in rubber stoppered test tubes at temperatures ranging from 25 C to 39 C for five weeks Still more remarkable results were recorded by Campbell Renton (1942) who drying single drops of peptone water cultures of cholera and El Tor vibrios *in vacuo* over phosphoric oxide (P_2O_5) still obtained positive results when making subcultures from six out of seven *V cholerae* and three out of five El Tor specimens tested after four years storage *in vacuo* at room temperature. In contrast to this favourable experience Shaw (1956) as well as some earlier workers mentioned by her reported that attempts at preserving cholera cultures with the aid of the spin freeze method of Greaves (1944) or of similar procedures gave no satisfactory results However according to the experiences of several observers (Burrows et al., 1947 Hornibrook, 1949 1950 Sokhey 1949 Sokhey & Habbu, 1950) freeze-drying (lyophilization) is an excellent means of preserving not only the viability but also the immunogenic properties of the *V cholerae* While Hornibrook (1950) recommended a menstruum containing only lactose citrates and inorganic salts as the best for freeze-drying bacteria or viruses, Neogy & Lahiri (1956) stated that skimmed milk was a good suspending medium for the preservation of *V cholerae* by the freeze-drying method but that the duration of preservation was never very long.

within 5-10 minutes if incorporated in mercury perchloride dilutions of 1 in 2 or 3 millions

Other antiseptic substances

As will be gathered from the summaries of Kolle & Schürmann (1912) and Mackie (1929) as well as from more recent publications in addition to the usual disinfectants a considerable number of other substances endowed with antiseptic properties have been found to exert an inhibitory or lethal action on *V cholerae* The following deserve mention

Soap The conclusions reached by Jolles (1893) that various sorts of soap were endowed with vibriocidal power were not confirmed by Murillo (1912) in whose experience addition of soap to nutrient media even in a concentration of 1/10 did not inhibit the growth of *V cholerae* Kolle & Prigge emphasized therefore that "even most thorough washing with soap was incapable of destroying the cholera vibrios"

Alcohol As maintained by Babes (1885) the maximal concentration at which alcohol added to nutrient media did not inhibit the growth of *V cholerae* was 1/15 It is in accord with this observation that as established by Van Ermengem (1885) broth cholera cultures became sterile within half an hour if absolute alcohol was added at a proportion of 1 10

Iodine In the experience of Babes (1885) addition of iodine to nutrient media at a concentration of 1/600 to 1/800 was just incapable of inhibiting the growth of *V cholerae* Bujwid (1892) found that iodine vapours retarded the growth of this organism but established in accord with previous experiences of Neisser (1887) and Riedlin (1888) that *Iodoform* exerted a far more marked action in this respect. Since various cholera like vibrios tested by Bujwid with iodoform were far less inhibited in their growth than *V cholerae* he suggested that this fact might be used in differential diagnosis—a proposal which is now interesting merely from the historical point of view

Potassium permanganate In contrast with the statement of Babes (1885) that potassium permanganate did not inhibit the growth of *V cholerae* Panja & Ghosh (1943) maintained that this chemical was lethal to cholera vibrios and still more to cholera like vibrios and that, therefore, "fruits and vegetables artificially infected with cultures of *V cholerae* can be effectively disinfected by soaking them in permanganate solutions of 1/5000 to 1/10 000 dilutions for 5 minutes" Since however this conclusion is not in accord with Babes observations and also not with the results of recent experiments made with other organisms such as *S typhosa* one should—as justly stated by the editor of the *Tropical Diseases Bulletin* (1943)—be cautious in accepting the recommendation of Panja & Ghosh until their results are confirmed by further tests.

that hydrochloric acid or sulfuric acid kills the vibrios in a few seconds if used in a concentration of 1/10 000 and lactic acid produces this effect even in weaker concentrations.

In the course of a study on the viability of the cholera vibrios in milk curd, which will be referred to later, Panja & Ghosh (1945) found that, besides hydrochloric acid and lactic acid acetic acid was also immediately fatal for these organisms if present in peptone water at a concentration sufficient to produce a pH of 4 4 However the vibrios were capable of surviving for five minutes if instead of these acids, citric acid was used under analogous conditions.

Interesting observations on the action of gastric juice on the cholera vibrio have been made by Napier & Gupta (1942) Whereas the vibrios added to a specimen of gastric juice taken from a patient who suffered from hyperacidity were killed immediately the organisms were apt to survive up to 24 hours (in one sample even up to 264 hours), if gastric juice from a patient with hypochlorhydria was used for analogous tests. Generally speaking, the vibrios survived for considerable periods (24 hours to maximally 370 hours) in specimens of gastric juice from which free hydrochloric acid was absent (pH 6 0 8 0) but succumbed immediately if the pH of the gastric juice was less than approximately 4 75 owing to the presence of free acid. However since Napier & Gupta found that addition of distilled water to the specimens tested prolonged the life of the vibrios in spite of a high initial acidity they believed that cholera vibrios ingested with a copious draught of water might pass the stomach in viable form even though large amounts of free hydrochloric acid were present. As pointed out by Greig (1929) when referring to earlier observations on the adverse action of gastric juice on *V cholerae* vibrios enclosed in a mass of food might also pass the stomach unharmed.

It may be conveniently added that, as found by Dawson & Blagg (1948 1950), in addition to normally acid gastric juice the saliva of healthy persons appears to exert an antibacterial action on *V cholerae* and might thus form a first line of defence against a not too massive infection

Disinfectants

As was early noted by Koch (1885) and confirmed by ample later observations, the usual disinfectants, even if used in low concentrations, exert a rapidly lethal action on *V cholerae* in suspensions or fluid cultures and inhibit the growth of this organism if added in small amounts to solid media destined for its cultivation. Thus Koch and his co-workers observed that phenol (carbolic acid) if used in a concentration of 0 5 / killed the vibrios in 10 minutes while at a concentration of 1 %, the organisms were killed in 5 minutes. Babes (1885) found that mercury perchloride, if added to gelatin at a concentration of 1/15 000 prevented the growth of the cholera vibrios, while according to Forster (1893) these organisms were killed

them in the presence of still lesser amounts of these dyes the organisms could be made dye fast, the strains then becoming capable of tolerating the action of the dyes at much higher concentrations

Exploring the possibility of incorporating aniline dyes into agar media destined for the cultivation of *V cholerae*, Signorelli (1912) found that addition of dahlia, erythrosin orcein or safranin led to a loss of virulence of the cholera vibrios developing on such media, which became decolorized while the colonies became intensely coloured Growth of *V cholerae* on agar containing methyl green or azolitmin also led to a decolorization of the media but the colonies not taking up these dyes, no loss of virulence resulted

Further interesting observations on the vibriostatic and vibriocidal properties of aniline dyes were made by Panja & Ghosh (1943) whose most important findings may be summarized thus

(*a*) Brilliant green, crystal violet, methylene violet, added to agar in a concentration of 1/100 000 exerted a bacteriostatic effect on *V cholerae* and El Tor vibrios. The same result was obtained with 1/50 000 concentrations of malachite green, acriflavin, gentian violet, methyl violet, methylene blue, fluorescein and pyronin yellowish, with thionin at a concentration of 1/25 000 with mercurochrome safranin, and basic fuchsin in concentrations of 1/5000 respectively

(*b*) Brilliant green and malachite green, if incorporated into peptone water at concentrations of 1/100 000 exerted a selective bactericidal effect on most cholera strains as well as on a large number of " paracholera " strains isolated from patients with clinical signs of choleraic disease, but these dyes did not affect the cholera like vibrios from river water

(*c*) Added in a final dilution of 1/5000, brilliant green killed the vibrios in cholera stools. These vibrios also disappeared earlier than in untreated cases from the stools of cholera patients who had been given the dye orally but clinical improvement was not marked. Since it had been found that an excess of alkali prevented the bactericidal action of the dyes *in vitro* this comparative failure of treatment probably stood in connexion with the alkaline reaction prevailing in the intestines of the patients.

Animal charcoal

Kraus & Barbará (1915a, 1915b) claimed that it was possible to render water free from cholera vibrios by shaking it with animal charcoal or by filtering it through a layer of this adsorbent. As they finally stated, it sufficed to shake 100-ml quantities of water which had been slightly contaminated with cholera vibrios after addition of 1 g of charcoal for 15 minutes However Kolle & Prigge (1928) commenting on these findings, rather doubted that this method of treating drinking water supplies could ensure complete sterilization.

Lime and milk of lime

Studying the action of various lime preparations on cholera vibrios Liborius (1887) found that

(1) A watery solution, containing 0 0246% of lime was capable of destroying the organisms within a few hours

Copper sulfate Copper sulfate, found effective against *V cholerae* in a concentration of 1/600 by Van Ermengem (1885) and in higher dilutions by Babes (tolerance limit 1/3000-1/5000) was recently again recommended by Halawani & Omar (1947) who found that this compound

"is lethal in dilutions ranging from 20-45 parts per million to *Vibrio cholerae* in concentrations ranging from 10 to 1 000 million per cc. of Nile water"

It is of interest to add that Bose & Chakraborty (1948) found metallic copper to be vibriocidal In the presence of strips of copper foil *V cholerae* could not be recovered from suspensions in distilled water in which the vibrios normally survived for two days In filtered tank water the presence of copper foil shortened the life of the organisms to about 1½ hours as against a survival for 15 days in the controls

Water which had been in contact with copper foil for periods of two or four hours also proved bactericidal within 30 minutes and 15 minutes respectively if cholera vibrios were added subsequent to the removal of the metal However no bactericidal effect was noted if water which had been in contact with copper foil for 48 hours was used for the preparation of peptone water or Douglas broth, cholera vibrios cultivated in such fluid media remaining viable for 15 days

Since satisfactory results were also obtained in tests carried out without the use of copper foil in polished copper vessels Bose & Chakraborty recommended the use of these containers during cholera epidemics for the temporary storage (4-6 hours) of water It is important to note in this connexion that chemical tests with the water samples used in these experiments failed to show the presence of copper

Essential oils

Since—as will be seen in Chapter 9—essential oils have been used with some success for the treatment of cholera it is of importance to note that in the experience of some workers such as Babes (1885) and Riedlin (1888) such oils were found capable of inhibiting the growth of *V cholerae* The former of these observers found mustard oil far more effective in this respect than peppermint oil oil of cloves bergamot oil or turpentine oil while Riedlin (who did not test mustard oil) found turpentine oil to be most antiseptic, followed in order of efficacy first by lavender eucalyptus and rosmary oil, then by oil of cloves Other essential oils including those of anise, fennel juniper peppermint, and thyme, were in Riedlin s experience of " subordinate importance "

Aniline dyes

Shiga (1913) found that some aniline dyes, particularly methylene blue and thionin, even if used in concentrations of 1/33 000 and 1/25 000 respectively inhibited the growth of cholera vibrios but that by growing

them in the presence of still lesser amounts of these dyes the organisms could be made dye fast the strains then becoming capable of tolerating the action of the dyes at much higher concentrations

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Lime and milk of lime

Studying the action of various lime preparations on cholera vibrios Liborius (1887) found that

(1) A watery solution containing 0 0246% of lime was capable of destroying the organisms within a few hours

(2) " Cholera broth cultures which contained numerous protein coagula and showed physical properties at least as unfavourable to the action of lime as cholera stools were permanently disinfected within a few hours through addition of 0 4% pure quicklime or 2% impure burnt lime in lumps "

(3) The lime was most active if it was used in the form of powdered pure quicklime or of 20% milk of lime prepared from the latter

Milk of lime the vibriocidal action of which in a concentration of 20% had been confirmed by Giaxa (1890) was according to Pfuhl (1892) pre scribed for the disinfection of cholera stools in an instruction (*Anweisung zur Desinfektion bei Cholera*) issued in 1892 by the Prussian authorities. According to this, one was advised to prepare milk of lime with 1 l of pure quicklime reduced to small pieces and 4 l of water to add the finished product to an equal amount of the stools to be disinfected and to let the mixture stand for at least one hour before disposal Since however, obser vers in Java claimed to have had bad results with this method in actual cholera work, Pfuhl made further investigations with fresh stools from patients in which *V cholerae* abounded He found the method effec tive, provided that the milk of lime was not merely poured over the stools but actually mixed with them. However prolonged stirring was unnecessary

Chlorine and chloride of lime

Making an early study of the disinfecting properties of chloride of lime, Niessen (1890) found that in pure cultures in the presence of 0 12% of this compound cholera vibrios usually became incapable of multiplica tion after one minute and invariably so after five minutes. Hence chloride of lime gave far more rapid results than quicklime, which, according to Liborius (1887) and Kitasato (1887-88) required at least one hour or even several hours to exert a disinfecting action.

Harding (1910) experimenting with water to which 1 2 drops of a 24 hours cholera culture had been added per litre concluded that most samples of contaminated water if treated with one part of chlorine per million for 15 minutes, are apt to be free from cholera vibrios How ever if organic matter is plentiful, it is advisable to use higher concen-trations, so as to leave, after oxidation of the organic matter 0.5-1 0 parts of chlorine per million available for the purpose of sterilization

Satisfactory experiments with chlorine compounds were made by Conor (1912) who found that cholera-contaminated samples of canal-water from Tunis were freed from the vibrios if chloride of lime or chloride of soda were added to the water at the rate of 2 mg per litre and allowed to act for eight hours. Conor considered it essential, however to utilize

freshly prepared solutions of these compounds with a content of free chlorine amounting to 30-40 per 1000

According to Langer (1913) addition of 0 5 g of chlorinated lime (corresponding to about 0 12 g of free chlorine) per litre of water sufficed to kill cholera vibrios and other pathogenic bacteria particularly if the desinfectant had been mixed with equal parts of sodium chloride to promote an even distribution Similarly favourable results were also recorded by Ditthorn (1915) who worked with a proprietary chlorine preparation

In contrast to the experiences mentioned above Genevray (1940a) laid stress upon the fact that in peptone water the cholera vibrio was found to resist considerable doses of free chlorine since an excess of 2 mg of free chlorine per 10 ml of the medium (i.e , 200 mg of free chlorine per litre) did not destroy it. Genevray found it pertinent to ask therefore what action chloride of lime used in Indochina for the disinfection of ponds during cholera epidemics could exert under these circumstances To answer this question it would be certainly desirable to make further investigations on the action of chloride of lime on *V cholerae* in areas where cholera prevails

Ozone

Investigating the value of ozone for water sterilization Schubert (1914) found this method preferable to that of sand filtration for rendering the water supplies free from cholera vibrios and from typhoid bacilli

Symbiosis

Voicing an often-expressed opinion, Kolle & Schürmann (1912) maintained that

"*In the case of a simultaneous presence of bacteria causing decay or rapidly growing* saprophytes no appreciable development of the cholera vibrios takes place under most natural conditions—indeed in most instances decay and decomposition are factors which rapidly destroy the cholera bacteria " [Trans.]

In support of this contention Kolle & Schürmann pointed out that in the experience of Koch (1885) and subsequent workers, as a rule the cholera vibrios did not survive long in highly contaminated and decaying substrates such as the contents of cess-pools or sewers

That also under other circumstances the presence of other bacterial species was apt to handicap or even to shorten the existence of *V cholerae* has been shown by (*a*) the observation of Rosenthal (1910) to the effect that cholera vibrios were unable to grow in milk or other suitable media in the presence of *Lactobacillus bulgaricus* which produced a markedly acid reaction and (*b*) investigations by Panayotatou (1913) demonstrating in the water of the Nile the presence of four (not further identified) bacterial species which were markedly antagonistic to *V cholerae* and thus

(2) "Cholera broth cultures which contained numerous protein coagula and showed physical properties at least as unfavourable to the action of lime as cholera stools were permanently disinfected within a few hours through addition of 0 4% pure quicklime or 2% impure burnt lime in lumps"

(3) The lime was most active if it was used in the form of powdered pure quicklime or of 20% milk of lime prepared from the latter

Milk of lime the vibriocidal action of which in a concentration of 20% had been confirmed by Giaxa (1890) was according to Pfuhl (1892) prescribed for the disinfection of cholera stools in an instruction (*Anweisung zur Desinfektion bei Cholera*) issued in 1892 by the Prussian authorities. According to this one was advised to prepare milk of lime with 1 l of pure quicklime reduced to small pieces and 4 l of water to add the finished product to an equal amount of the stools to be disinfected, and to let the mixture stand for at least one hour before disposal. Since, however observers in Java claimed to have had bad results with this method in actual cholera work Pfuhl made further investigations with fresh stools from patients in which *V cholerae* abounded. He found the method effective, provided that the milk of lime was not merely poured over the stools but actually mixed with them However prolonged stirring was unnecessary

Chlorine and chloride of lime

Making an early study of the disinfecting properties of chloride of lime, Niessen (1890) found that in pure cultures in the presence of 0 12% of this compound cholera vibrios usually became incapable of multiplication after one minute and invariably so after five minutes. Hence chloride of lime gave far more rapid results than quicklime, which, according to Liborius (1887) and Kitasato (1887-88) required at least one hour or even several hours to exert a disinfecting action.

Harding (1910) experimenting with water to which 1 2 drops of a 24 hours cholera culture had been added per litre concluded that most samples of contaminated water if treated with one part of chlorine per million for 15 minutes, are apt to be free from cholera vibrios However if organic matter is plentiful, it is advisable to use higher concentrations, so as to leave, after oxidation of the organic matter 0.5-1 0 parts of chlorine per million available for the purpose of sterilization

Satisfactory experiments with chlorine compounds were made by Conor (1912), who found that cholera-contaminated samples of canal-water from Tunis were freed from the vibrios if chloride of lime or chloride of soda were added to the water at the rate of 2 mg per litre and allowed to act for eight hours. Conor considered it essential, however to utilize

Viability of V. cholerae Outside the Body[1]

Faeces

Describing the experiments made by Koch and his co-workers during their initial work, Gaffky (1887) stated that

"if intestinal contents or faeces rich in cholera bacilli but containing other bacteria as well were put on moist earth or linen and were kept in a manner preventing exsiccation at first the cholera bacilli grew most luxuriantly so that after 24-48 hours specimens taken from the surface contained—as proved by microscopic examination—the cholera bacilli practically in pure culture. However after a few days already they began to die and the other bacteria started to multiply" [Trans.]

Though Koch and his co-workers were unable to state definitely what the maximal period of survival of the vibrios under these and similar conditions was their observations indicated that—particularly if decomposition took place and/or other bacteria were present—the *V. cholerae* did not persist long.

While Koch and his co-workers made their observations with what Greig (1914a) afterwards called "uncultivated" strains of *V. cholerae*, i.e., *directly with the faeces of cholera sufferers* many of the numerous other workers investigating the survival of this organism used for their tests the dejecta of individuals free from cholera to which they added cultivated vibrios While one must fully agree with Abel & Claussen (1895) Greig (1914a) and Gildemeister & Baerthlein (1915) who made the most valuable investigations in this field, that only observations with cholera faeces can be considered valid it is interesting to see that they adduced different reasons why the results obtained with artificially infected stool specimens should be rejected Abel & Claussen (1895) maintained in this connexion that quite possibly the "comma bacilli" in the cholera faeces where they are often present in almost pure culture possessed a higher vitality than those in artificial stool mixtures, where they were subjected to competition with other bacteria. Greig (1914a) on the contrary recommended "uncultivated" material because experiments previously made with typhoid bacilli seemed to indicate that stock cultures of *V. cholerae* were more resistant than the vibrios in the faeces. Gildemeister & Baerthlein (1915) stressed with much reason the importance of results with rice water stools because owing to the abundance of mucous material, these were less prone to undergo exsiccation than other kinds of faeces

Several of the early workers who could examine genuine cholera stools in Europe found that the average period of persistence of the vibrios was longer than the preliminary findings of Koch and his co-workers in India had indicated, and some reported instances of an excessively long survival of the organisms While in view of the limitation of the differential diagnostic methods available to the early observers the latter records must be inter

[1] The occurrence and persistence of cholera vibrios in animals (e.g., in flies and aquatic animals) will be dealt with in later parts of this book

apparently responsible for the failure of the latter to persist under laboratory conditions in water samples from that river

It is important to note, however that symbiosis with other bacterial species was by no means always found to be unfavourable to the persistence of *V cholerae* Kabelik & Freudmann (1923) noted in this connexion that, when cultivated in peptone water together with *E. coli* cholera vibrios grew far more luxuriantly than did *E. coli* Sarkar & Tribedi (1953) again studying the relation between these two organisms found that when a loopful of a cholera culture was added to a 24-hour-old broth culture of *E. coli* and daily platings were made during an initial period lasting from three to 14 days *E coli* colonies only grew on the plates Subsequently however cholera colonies appeared in increasing proportions, to become finally solely present. Sarkar & Tribedi found that this evolution was paralleled by changes in the pH of the broth culture the preliminary cultivation of *E. coli* led, after 24 hours, to a lowering of the initial pH of 7 6 to 7.2. After addition of the cholera vibrios the pH rose, vibrio colonies appearing as soon as it had reached 8 8 and being solely present, when the pH had reached 9.2. However in the opinion of the two workers, this rise of the pH alone was not responsible for the disappearance of *E. coli* because it was found that (*a*) prolonged cultivation of this organism alone in broth led to a pH of 9 0 at which it was able to survive and (*b*) it was viable for several days in broth with an initial pH of 9 8 which it lowered within 24 hours to 9.3 It was also noted that, while *E. coli* was unable to multiply in a broth culture in which cholera vibrios alone had survived it could multiply in these cultures if the vibrios had been killed through heating for one hour at 60°C. Sarkar & Tribedi postulated, therefore that the antagonistic action exerted by *V cholerae* and (as they also established) by cholera like vibrios on *E. coli* was due to the presence of a thermolabile colicidal substance The antagonism exerted by the cholera vibrios was also manifest if they were present in stools together with *E. coli*

Carrying out exhaustive and exact studies on the effect on *V cholerae* of the concurrent presence not only of *E. coli* but also of *Aerobacter aerogenes* of *Proteus vulgaris* of *Streptococcus faecalis* a Gram positive coccus isolated from water and of water vibrios not agglutinable with cholera-immune serum, Read et al. (1939) established that under these circumstances

"the agglutinable vibrio can survive in weak peptone water and salt solutions even when present in smaller inoculum, except in the presence of certain inagglutinable vibrios. In several experiments it survived for two weeks or more in the presence of the latter vibrios."

The observations recorded above as well as those to be dealt with now show that the cholera vibrio is by no means as invariably frail an organism as it is assumed to be by some authorities

Month of examination	*Number of stools examined*	*Duration of life of cholera vibrios (days)*			*Average temperature*
		minimum	*maximum*	*average*	
December 1912	9	1	10	3 6	
January 1913	6	1	12	6 6	72°F (22°C)
February	13	3	17	7 7	
March	20	1	13	6.5	
April	22	1	5	2.8	
May	10	1	3	1 4	85 F (29.5°C)
June	15	1	2	1.2	
July	—	—	—	—	
August	4	1	12	6.0	
September	3	4	5	4.3	83 F (28°C)
October	3	3	4	3 7	

Greig concluded from these investigations that though there was con siderable variation between individual strains "the life of the cholera vibrio outside the human host under natural conditions in India is short" and added the equally important statement that

"Temperature has a powerful influence on the vitality of the cholera vibrio outside the human host. Thus as the hot season, in Calcutta from March to June advances, the life of the organism becomes shorter In the present research the minimum duration of life was reached in June. On the other hand, from December to February the cold season, the vitality is greater and the maximum duration of life occurred in February Again in August when the monsoon has fully developed and the temperature has fallen somewhat the life is longer than in the hot season but as the number of cases of cholera during August and September is small my observations during this period were fewer"

That the prevailing temperature exerts an important influence on the period of survival of *V cholerae* in the faeces of the patients, was also shown by observations in Japan In addition to findings recorded in this respect by Takano, Ohtsubo & Inouye (1926) Soda et al. (1936) noted that if specimens of one and the same cholera stool were kept at 37°C, at room temperature and in the ice box respectively, survival of the vibrios was longest (up to eight days) in the last case shortest (sometimes only 3 hours) at body tempe rature (37 C) Identical experimental observations had been previously recorded by Shoda, Koreyada & Otomo (1934)

These observations as well as the fine studies of Greig leave little room for doubt that differences in the prevailing temperature were largely if not solely responsible for the marked differences observed in regard to the length of survival of the causative organisms in cholera stools in India and Europe respectively It is significant that analogous differences were noted when the length of survival of the cholera vibrios in sewage cesspools or septic tanks and the like was studied. Flu (1921a) established in this con nexion that, in contrast with *S typhosa* cholera vibrios persisted in the septic tanks of Batavia not, or not much longer than 24 hours. In Europe on the contrary to judge from the summary of Fürbringer & Stietzel (1908) sur vival periods of *V cholerae* in cesspools manure and the like for one to two weeks have been recorded by several observers. That in these cases

preted with great caution, the fully reliable findings of Abel & Claussen (1895) and of Gildemeister & Baerthlein (1915) deserve great attention

Abel & Claussen (1895) worked with 31 cholera stools which had been sent for diagnostic purposes to the Institute of Hygiene in Königsberg in Prussia. Once the diagnosis of cholera had been established, these samples, kept in the well-closed bottles in which they had arrived, and protected against direct sunlight, were stored at room temperature (13°-16°C). Re-examinations with the aid of peptone water enrichment were made daily or at least every few days. If loopfuls of the stools gave no results, larger amounts up to 50 ml were used for peptone water enrichment and platings and only if these also proved negative, were the cholera vibrios considered to have disappeared.

Abel & Claussen found under these circumstances a persistence of the cholera vibrios for 1-5 days in eleven instances, for 6-10 days in six, up to 15 days in nine, for 15-17 days in three, for 24 and for 29 days in one instance respectively. It will be noted, therefore, that (*a*) the cholera vibrios disappeared from about one-third of the samples within five days and (*b*) that a survival of the organisms for more than 15 days was not frequent (16.1 %).

Working during the First World War at Posen (now Poznan), Gildemeister & Baerthlein (1915) examined 70 stools derived partly from cholera patients, partly from supposedly healthy carriers, only few of the samples showing a typical rice-water-like appearance. Their technique differed in some details from that of Abel & Claussen, particularly because (*a*) they kept their specimens (protected from direct sunlight and at room temperatures ranging from 12°C to 21°C with an average of 18°) in covered Petri dishes, so that they were apt to undergo exsiccation and (*b*) they used for their platings blood alkali agar as recommended by Dieudonné (1909), whereas Abel & Claussen had worked with gelatin plates.

The results obtained by Gildemeister & Baerthlein may be summarized thus

Period of survival (days)	*Number of specimens*	*Percentage*
1	18	25.7
2-5	8	11.4
6-10	9	12.8
11-15	8	11.4
16-20	11	15.7
21-30	11	15.7
31	1	
34	1	
36	1	7.1
37	1	
51	1	
Total	70	99.8

Gildemeister & Baerthlein concluded, therefore that

"(1) Cholera vibrios succumb in a major part of the cholera stools within a short time

(2) However in a not inconsiderable part of the stools they remain viable for several weeks, sometimes more than 30 days." [Trans.]

Greig (1914a) using for his observations on the persistence of *V cholerae* 94 typical cholera stools freshly collected in Calcutta and applying a technique similar to that of Abel & Claussen, but taking advantage of Dieudonné as well as of agar plates, recorded the following results

Month of examination	*Number of stools examined*	*Duration of life of cholera vibrios (days)*			*Average temperature*
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July	—	—	—	—	
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The results obtained by Gildemeister & Baerthlein may be summarized thus

Period of survival (days)	*Number of specimens*	*Percentage*
1	18	25 7
2 5	8	11 4
6-10	9	12.8
11 15	8	11 4
16-20	11	15 7
21 30	11	15 7
31	1	7 1
34	1	
36	1	
37	1	
51	1	
Total	70	99 8

Gildemeister & Baerthlein concluded therefore, that

"(1) Cholera vibrios succumb in a major part of the cholera stools within a short time

(2) However in a not inconsiderable part of the stools they remain viable for several weeks, sometimes more than 30 days." [Trans.]

Greig (1914a) using for his observations on the persistence of *V cholerae* 94 typical cholera stools freshly collected in Calcutta and applying a technique similar to that of Abel & Claussen but taking advantage of Dieudonné as well as of agar plates, recorded the following results

Studying the possibilities of aerial transmission of infectious diseases Germano (1897) exhaustively experimented with different dusts to which cholera vibrios in suspensions or incorporated in normal faeces had been mixed He found that the vibrios survived well if the dust was kept moist but died rapidly (maximally within three days in the case of brick-dust) if exsiccation took place Germano concluded therefore that the chances of transmission of cholera through the air were " extremely slight "

It may b conveniently added that, according to the summary of Takano Ohtsubo & Inouye (1926) investigations on the survival of *V cholerae* on coal have been made by Hata & Matsuda (1906) These two workers noted that the organisms persisted in a mass of moistened coal for seven days and in a mass of dry coal for 21 days moisture apparently hastening the death of the organisms by facilitating the growth of other bacteria

Cloth and cotton A few workers, e g. Gamaleia (1893) and Karliński (1895) found that under highly artificial conditions counteracting exsic cation the cholera vibrios on pieces of cotton or cloth which had been soaked in suspensions of these organisms could survive for several weeks or even months. However most observers who performed their tests under conditions comparable to those actually obtaining agree that the cholera vibrios persist on these contamined materials for a few days at the most (one to five days according to the summary of Jettmar 1927) The earlier observations made to this effect have been confirmed by those made during the 1947 cholera outbreak in Egypt, when as recorded by Shousha (1948) it was shown by tests with faeces of the cholera patients that *V cholerae* survived on depurated cotton for two days only and on raw cotton and cloth for three days.

Leather and rubber According to Gohar & Makkawi (1948) the causa tive organisms in the stools of cholera patients survived for two days on leather but for not more than six hours on rubber

Paper To judge from the scanty information available the survival period of *V cholerae* on paper or objects made of paper is short. Uffel mann (1892) stated in this connexion that if cholera stools were left to dry on the printed page of a book (which took about 10 minutes) and the book was then closed and kept in a cupboard, the vibrios survived for at least 17 hours. Under the same conditions the organisms remained viable for at least 23½ hours on letter paper enclosed in an envelope and for at least 20 hours on a postcard

Germano (1897) found that the *V cholerae* was able to survive for one day only on blotting paper which was let to dry after it had been soaked in a suspension of the organisms If exsiccation was prevented the vibrios were still viable on the 20th day

Tests with Chinese bank notes which had been handled by fingers con taminated with cholera stools showed that, after the bank notes appeared

temperature differences also played an important role is suggested by observations of Ohwada (1924) which showed that *V cholerae* survived in sewage at 37°C for one day at room temperature for four days, and in the ice-chest for twelve days.

Dead bodies

Dunbar (1896) the first and it would seem the only worker to try to determine the length of survival of the causative organisms in cholera victims, was able to examine the dead bodies of 10 individuals who had been buried during the 1892 outbreak at Hamburg. Most of these persons had been buried in September and one at the end of December and they had been exhumed during the period from December 1892 to April 1893 It was impossible to find cholera vibrios in any of these bodies which included (*a*) one buried on 1 September and exhumed on 5 December and (*b*) another buried on 25 December and exhumed on 6 April of the following year It is curious that the intestinal contents of the latter victim, obtained at autopsy and kept in the laboratory at room temperature still proved positive for *V cholerae* at the time of the exhumation of the body and for two weeks afterwards

In view of these findings made during autumn and winter in Europe it is altogether unlikely that the causative organisms survive for any considerable length of time in the dead bodies of individuals succumbing during cholera outbreaks which typically take place during the warm season in the countries usually affected

Contaminated material

Besides the work of Koch and his co-workers, whose experiments with moist earth and linen were referred to above, the fate of *V cholerae* in these and other substrates contaminated with faeces or with material from cultures has been studied by numerous other workers. While the experience they gained with foodstuffs will be dealt with separately below the following findings obtained with other materials deserve attention

Earth and dust Nicati & Rietsch (1885) claimed that, if cholera stools were sprinkled on moist earth, the vibrios remained viable for 14-16 days However Uffelmann (1893b) concluded from numerous experiments that *V cholerae* if added at high concentration to samples of garden earth, survived at room temperature for two to three days only The viability of the organisms could be prolonged to 12 days if the samples were kept at 6 C and to 16 days at 0 C to + 1 C, i.e., at temperatures hardly ever met with in the regions where cholera is prevalent. In fact Flu (1915) found that in Batavia, Java, the length of survival of the causative organisms in the rice water stools of cholera patients poured on the ground did not exceed seven days even if the weather was moist.

for three to four days, but that storage of the material in the ice box prolonged the viability of the organisms to 10-12 days

As quoted by Takano and co-authors (1926) Toyama working mainly with freshwater fish established that cholera vibrios smeared on the meat of these animals remained viable for two to three days during midsummer for seven to ten days in early summer for one to two weeks in mid winter Storage of the fish in an ice box at 3°-8°C prolonged the survival period of the vibrios to 14-19 days, occasionally even to 25 days

Toyama further stated that when oysters or clams were kept in cholera polluted sea water the vibrios rapidly entered their gastro-intestinal tract and survived there for 1½ months at a temperature of 0 5 C, and for 15-20 days at 22°C. When cholera vibrios were smeared on shelled oysters kept at room temperature (about 20 C) the number of organisms first decreased but soon began to increase and reached a maximum in 68 hours, followed by a gradual decrease and disappearance of the vibrios in 171 hours *V cholerae* remained viable for 20 days if smeared on oysters or clams which had been killed and sterilized by boiling. As shown by tests with contamined oysters which had been soaked in dilute acetic acid it was comparatively easy to kill the cholera vibrios on their surface but the organisms survived in the intestinal tract of the oysters for seven hours when 1% 2% acetic acid was used for two hours in the case of 3% acetic acid and for 45 minutes if the concentration was increased to 4% 5%.

Milk In a classical study on the behaviour of *V cholerae* in milk, Kitasato (1889b) established the following periods of survival of the organisms

Temperature	*Raw milk*	*Raw milk with 10% sodium carbonate*	*Steam-sterilized milk*
36°C	14 hours	55 hours	2 weeks
22° 25°C	1 1½ days	Still fairly numerous after 78 hours	Still viable after 3 weeks
8° 18°C	2 3 days		

Noting an incessantly progressing acidification of the milk media in the course of these tests, Kitasato emphasized that

" the length of survival of the cholera bacteria is dependent upon the reaction of the milk the more rapidly the milk sours, the more rapidly the cholera bacteria therein perish however the cholera bacteria survive until the milk becomes strongly acid " [Trans.]

Since heating of samples of raw or raw alkaline milk inoculated with cholera vibrios for five minutes at temperatures ranging from 96 C to 100 C rendered the samples sterile Kitasato also concluded that " boiling is the simplest and most effective method to free milk from cholera germs "

Most subsequent observers confirmed that under the ordinarily prevailing temperatures cholera vibrios could survive in raw milk for at least

to be dry the vibrios remained alive for maximally four hours (Jettmar 1927) However recent observations in Egypt (Shousha, 1948) showed a survival period of *V cholerae* on bank notes contaminated with cholera stools for two days, and on postage stamps treated in the same manner for one day

Metals Uffelmann (1892) found that if cholera stools were put on copper and silver coins and permitted to dry the vibrios remained viable for 10-30 minutes only Identical results were obtained with brass plates.

Tests made during the 1947 cholera epidemic in Egypt with faeces-contaminated coins showed a survival of *V cholerae* for seven hours (Shousha, 1948)

Tobacco Wernicke (1892) found that even on moist cigars and snuffing tobacco cholera vibrios succumbed within 24 hours.

Food

For obvious reasons the fate of *V cholerae* in food materials contaminated with cholera stools or cultures has attracted the attention of numerous workers. Babes (1885) who seems to have been the first to make systematic studies in this respect, noted that the cholera vibrios remained alive up to 48 hours on fresh non-acid vegetables, potatoes, and cheese but not longer than 24 hours on sour fruit and vegetables.

Essential findings made by subsequent workers may thus be summarized

Meat There can be no doubt that under suitable environmental conditions meat and meat products form a favourable substrate for the survival of *V cholerae* Thus Uffelmann (1892) found that on roast pork, kept under a glass bell, the vibrios survived for at least eight days. Lal & Yacob (1926) testing various Indian foodstuffs, placed meat high among those found potentially suitable for the cultivation of the cholera vibrios. Japanese observers (see Takano Ohtsubo & Inouy 1926) found meat a suitable substrate for the survival and, during the first 20 hours after contamination, for the multiplication of the *V cholerae* Cholera vibrios on the surface of meat which was kept in the open during mid winter were found to be able to survive for one to two weeks.

Fish and shellfish As shown by numerous observations, fish and shellfish, stored pending consumption form a suitable substrate for quite prolonged survival of *V cholerae*

Systematic studies made by Friedrich (1893) showed that cholera vibrios were apt to survive on fresh fish for two days, on smoked herrings for one day (according to Uffelmann (1892) even for four days) on caviar for three to six days or if the latter was kept in the ice box, even longer

Takano (1913) found that cholera vibrios smeared on fish meat, which was then kept at room temperature during the month of October survived

TABLE XIX. PERIODS OF SURVIVAL OF V CHOLERAE IN SALT SOLUTIONS

Chemically pure NaCl				Common cooking salt
concentration (%)	37° C	room temperature	2°–4° C	– 4° C
1	1 month	1 month	10 days	15 days
5	10 days	1 week	15 hours	15 days
10	1 da	1 day	13 hours	3 days
15–25			7½ hours	2 days
Saturated solution				14 hours

Autumn season

Analogous investigations by Genevray & Bruneau (1938a) showed the survival of *V cholerae* in solutions of either NaCl or sea salt to be as follows

Concentration (%)	*Period of survival*
2.5	One month or more
5.0–7.0	More than three weeks
8.0	More than two weeks
9.0–11.0	24–48 hours

Since no multiplication of the vibrios was observable at salt concentrations exceeding 8% (80 per 1000) Genevray & Bruneau felt certain that sea-salt in bulk did not play a role in the spread of cholera.

However, though high salt concentrations exert an unfavourable influence on the survival of *V cholerae* Venkatraman & Ramakrishnan (1941) found 2% solutions of sea salt (or of the impure salt obtainable in the bazaars of India) in carefully buffered saline, prepared as will be described in Chapter 7 excellent vehicles for the transmission of cholera suspect stools to distant laboratories In the experience of these two workers, cholera vibrios in artificially contaminated stool samples remained viable in such solutions for 62 days, while the vibrios in actual cholera stools were preserved even up to 92 days, the pH of the solutions remaining at its original level of 9 2.

Sugar and honey Shousha (1948) noted that contamination of sugar with cholera stools resulted in a survival of the vibrios for three days To judge from tests made with cultures *V cholerae* survived for one day only on honey

Bread and cakes As noted by Uffelmann (1892) *V cholerae* was apt to survive for at least one day on slices of unwrapped rye bread, for up to three days on rye bread wrapped in paper for at least one week on bread kept under a glass bell.

one to two days, regardless of whether or not the milk became acid in the meanwhile or as some workers such as Heim (1889) and Basenau (1895) expressly stated, even regardless of whether or not the milk had curdled. It is, however interesting to note that in the experience of Panja & Ghosh (1945) cholera vibrios added to Indian milk-curd (*dahi*) were killed within five minutes and that according to recent observations in Egypt (Shousha, 1948) the life-span of *V cholerae* added to already sour milk was only one hour These observations seem in accordance with experimental findings of Heinemann (1915) who established that cholera vibrios were immediately killed if added to samples of sterilized milk which contained 0 45% lactic acid or as seems indicated by the protocols of this worker even at lower concentrations of the acid.

It is, on the other hand, important to realize that boiled milk, if contaminated by *V cholerae* pending storage is at suitable temperatures a substrate favourable for the initial multiplication and survival of this organism. Observations made in this respect in the Berlin Gesundheitsamt (1892) showed that whereas cholera vibrios added to raw milk survived for less than 24 hours, they remained viable for nine days in milk which had been boiled for one hour and again cooled before contamination.

Whey According to Heim (1889) whey even though its originally alkaline reaction had turned weakly acid, was still positive for *V cholerae* on the second day following contamination

Butter Heim (1889) found that cholera vibrios, while surviving for a day only on low-grade slightly acid butter remained viable on butter of better quality for over one month. However other observers, such as Laser (1891) and Uffelmann (1892) recorded periods of survival of *V cholerae* on butter not exceeding one week.

Cheese As stated by Babes (1885) and some subsequent European observers, cholera vibrios were apt to remain viable on cheese for 48 hours. Shousha (1948) in Egypt noted a survival period of the organisms on "white" cheese (probably a local product) for only two hours. An interesting parallel to this observation is that, according to Heim (1889) cottage cheese gave positive results only immediately after contamination with *V cholerae*

Salt As maintained by Takano Ohtsubo & Inouye (1926) experiments carried out in Japan had shown that the cholera vibrios do not multiply in salt solutions and are even gradually killed. The data they furnished to support this contention are summarized in Table XIX. They added that

" In salting fish, if impure salt be used and left at a room temperature, the cholera vibrios survive for 2 weeks. The effect is better if the abdominal viscera of the fish be removed and the fish be packed in salt."

Trasler (1922) in Mesopotamia for three days and according to observations made in the Berlin Gesundheitsamt (1892) even for 5-7 days For, as quoted by Sticker, Hankin (1896b) not only incriminated cucumbers as being instrumental in the causation of some cholera cases in India but supported this contention by demonstrating the presence of *V cholerae* on the cucumbers in question There can be no doubt that the use of human manure for fertilizing cucumbers, which are often eaten uncooked renders them potentially rather dangerous for the transmission of cholera

Fruit Systematic investigations made with a series of different fruits and berries in the Berlin Gesundheitsamt (1892) yielded rather variable results with survival periods of *V cholerae* ranging from one hour to between three and seven days at room temperature for somewhat shorter periods (up to four days) at 37°C. It was noted that the organisms could survive on the surface of dried European fruit for one to two days

The results obtained under somewhat unrealistic conditions by Pollak (1912) may thus be compared with recent experiences made with cholera stools during the 1947 outbreak in Egypt

Kind of fruit	*Pollak (1912)[a]*	*Shousha (1948)*	
Apples	16 days	—	
Dates	—	Outside	2 days
		Inside	3 days [b]
Grapes	— [c]	Outside	2 days
Lemons	14 days	Skin	3 hours
		Inside	1 hour
Oranges	10 days	Skin	3 hours
		Inside	1 hour

[a] Maximal periods observed.

[b] Gohar & Makkawi (1948) whose statements often do not tally with those of Shousha asserted " that dates contaminated from outside could act as vehicles of infection for as long as four days and that the organisms could not survive long in the inside probably on account of the splitting of carbohydrates resulting in the production of acidity "

[c] Pollak quoted Dobroklonski (1910) to the effect that *V cholerae* while surviving inside the berries for not longer than 24 hours, could persist on the outside of grapes for four days, and on their stalks for even 12 days.

Since as will be discussed later in China at least cut melons have been found to play quite an ominous role in the transmission of cholera infection it is important to note that according to the laboratory observations of Friedrich (1893) the inside of these fruits was an excellent substrate not merely for the survival but for the multiplication of *V cholerae* as long as exsiccation could be prevented It has to be added however, that according to the experience of Mackie & Trasler (1922) in Mesopotamia cholera vibrios were able to survive for only three days on melons which had a strongly acid reaction at all stages of ripening.

According to Friedrich (1893) cholera vibrios survived on pastry not longer than 24 hours but were able to persist on biscuits for periods up to four days.

Cereals Observations made during the 1947 outbreak in Egypt (Shousha 1948) showed a *V cholerae* survival of two days on rice and lentils contaminated with the stools of patients. A much shorter survival (7 hours) was noted in tests made with cholera cultures, thus lending support to the assumption that the organisms in the actual stools are better protected against adverse conditions than those grown on artificial media

There can be no doubt that, as summarized by Sticker (1912) rice gruel and similar dishes prepared from cereals, if kept under suitable temperatures, form a favourable substrate for the growth of *V cholerae*

Potatoes As summarized by Sticker cholera vibrios were apt to survive on the surface of raw potatoes for at least 48 hours. The acid reaction initially present on the cut surfaces of potatoes was unfavourable for the organisms, but in the case of some kinds of potatoes a change to an alkaline reaction could take place spontaneously which favoured the persistence or even the multiplication of *V cholerae* Sticker added that cold potato dishes were a favourable substrate for this organism which could multiply there without causing visible changes.

Onions and garlic Contrary to the popular belief that their consumption is apt to confer protection against cholera infection, onions and garlic actually form fairly good substrates for the survival of *V cholerae* Tests carried out in this respect with faeces of patients during the 1947 outbreak in Egypt showed according to Shousha (1948) the following survival periods

Onions	— outside	2 days	Garlic	— outside	1 day
	— inside	3 days		— inside	2 days

Green vegetables While according to earlier experiments, such as those of Babes (1885) and Uffelmann (1892) cholera vibrios were able to persist on green vegetables for two to three days only longer periods of survival (up to 22 days on spinach, even up to 29 days in the case of one lettuce specimen) have been recorded by Pollak (1912) Though it has to be noted that the conditions under which this worker experimented did not correspond well to those actually prevailing, he was certainly right in stressing that persistence of an adequate degree of moisture was apt to promote a prolonged survival of *V cholerae* on green vegetables (and also on fruit)

It is important to note that cholera vibrios can survive on cucumbers, which have a mildly acid reaction, for some days—as found by Mackie &

Writing in 1921, Rogers stated without furnishing an adequate reference that

"Hankin showed some years ago that the cholera bacillus dies out of aerated water within a few days, so unless this commonly consumed fluid can be obtained from a firm who can be relied on to sterilize the water employed in its manufacture, it is safer to keep it for several days before consuming it, if cholera is about"

Making further studies on the survival of *V cholerae* in aerated drinks Yacob & Chaudhri (1945) found that the organisms, when added to soda water (pH 6 8) were no longer demonstrable after two hours, and also noted an absence of the vibrios 10 minutes after blocks of cholera infected ice had been added to samples of non-contaminated soda water These ice cubes had been prepared by freezing for 24 hours in a refrigerator one litre of water into which 7 ml of a 24-hour culture of *V cholerae* in peptone water had been incorporated previously

Survival in water

Fresh water While, as described in detail by Gaffky (1887) in the course of their work at Calcutta Koch and his colleagues were able to demonstrate the presence of *V cholerae* in a tank which served as a source of water supply in a cholera focus, they could not reach definite conclusions regarding the possibilities of survival and multiplication of cholera vibrios in water in general, because the few investigations they could make in this direction gave discrepant results

However ample observations made by other workers soon filled this gap Reviewing the results these investigators had obtained Gotschlich summarized in 1903

(*a*) Sterile distilled water was not a suitable substrate for the survival of *V cholerae* but addition of minimal quantities of nutritive substances or of NaCl created more favourable conditions.

(*b*) In the experience of some observers, the cholera vibrios could survive for prolonged periods in sterilized spring or well water according to Wolfhügel & Riedel (1886) for periods of up to a year

(*c*) On the contrary the survival of the organisms in the filtered and/or sterilized water supplied by water works was short, e.g., 7 days in Berlin tap-water according to Babes (1885)

(*d*) The periods of survival observed in the case of the raw water of wells and springs varied from about one day to several weeks (up to 10 weeks according to Kruse, 1894).

(*e*) The length of survival of *V cholerae* in raw river pond or tank water also varied within wide limits, being for instance, according to Cunningham (1889), not more than four days in the water of Calcutta tanks, varying according to Uffelmann (1892, 1893b) in the port and river water of Rostock from 1 to 20 days, amounting under peculiar conditions in the deposits at the bottom of an aquarium to three months (Wernicke, 1895).

Some of the early workers clearly recognized that the marked differences noted in regard to the persistence of *V cholerae* in different water samples

Local foods Lal & Yacob (1926) testing the relative suitability of certain foodstuffs used in India as substrates for the growth of *V cholerae*, recorded the following

(1) Articles containing salt and animal or vegetable proteins, such as meats, fresh fish, boiled rice, fresh milk, bazaar biscuits, oven-baked Indian bread, halwa, as well as radish, cooked greens, and water melons were specially suitable for the growth of *V cholerae*

(2) Contrary to popular beliefs, chillies and onions did not inhibit the growth of the vibrios.

(3) Sugared foodstuffs gave variable results.

(4) Fats proved poor culture media, while sour articles, such as pickles and beer were not likely to convey the infection.

Takano Ohtsubo & Inouye (1926) recorded the following results with Japanese condiments

"The [cholera] vibrio smeared on the body of fish is not killed in sugared vinegar for 2 hours. It survives for over 20 hours at room temperature in vinegar-bean-paste, and soy (soy-bean sauce) has even less disinfecting power than has vinegar-bean-paste. In short, it may be said that fresh fish prepared with vinegar bean paste or soy is dangerous food during a cholera epidemic."

As found by Genevray & Bruneau (1938b) in Indochina, soy bean milk and soya cheese in which cholera vibrios survived for 12 hours and which were consumed on the day of preparation, were dangerous food articles at the time of epidemics. The length of survival of *V cholerae* was less than one hour in fermented soya sauce four to five hours in prawn paste and three to six hours in an Annamite dish (*nuoc mam*) made from macerated fish, and since these articles were invariably stocked for some time before they were sold it seems unlikely that they played a role in the conveyance of cholera infection

Beverages

Babes (1885) maintained that cholera vibrios could stay alive in coffee, chocolate, and fruit juices for 48 hours and in beer and wine for less than 24 hours. Systematic investigations made in this direction in the Berlin Gesundheitsamt (1892 see also Friedrich, 1893) gave the following results

Drink	*Viability of V cholerae*	*Drink*	*Viability of V cholerae*
Beer	Up to 3 hours	Coffee with milk*	8 hours
Wine	5-15 minutes	Tea (1 %)*	8 days
Coffee (6 %)	2 hours	Tea (4 %)*	1 hour
Coffee with chicory*	5 hours	Cocoa (1 2 %)	7 days

Contaminated after cooling.

Panja & Ghosh (1945) found that undiluted lime juice (pH 2.8) killed cholera vibrios within five minutes, and lime juice freshly diluted with 1% peptone water (final pH 4 4) within half an hour

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were due either to variations in their character particularly their varying content of organic matter (Koch, 1884) or to extrinsic conditions, especially differences in the temperature of the water (Nicati & Rietsch, 1885) or to both these factors The great importance of the latter factor was demonstrated by Uffelmann (1892, 1893b) who found that cholera vibrios survived in the water of the Rostock (river) port for one day at 30°C, for two to three days at 20 C and for five days at 10°C. At a temperature of 6°C the vibrios were found to survive for at least 20 days in the river water and for at least 23 days in tap water

In marked contrast with the results obtained in the case of rivers in Europe Hankin (1896a) found that *V cholerae* could not persist even for two hours in the raw water of the Ganges and Jumna Rivers in India. Since boiled water samples no longer proved inimical to the growth of the organisms Hankin postulated that the raw water of these rivers exerted a vibriocidal action due to the presence of volatile acid substances.

That river water which is unsuitable for the persistence of *V cholerae* may also be met with in other areas where cholera is apt to prevail was shown by observations made later by Pollitzer (1934a) who found that cholera vibrios added to specimens of the Shanghai River and creek water were no longer demonstrable in 66% of the 50 samples tested, after they had been kept in the dark at room temperature for 24 hours. A survival of 24 hours was noted in 26% of the samples, longer periods of survival up to maximally four days being exceptional. There was no doubt that *the short persistence of the vibrios was due to the heavy contamination* of the Shanghai surface waters with various bacteria. Cholera vibrios added not only to sterilized but also to Seitz filtered specimens of the waters used for the above-mentioned tests persisted for months and in some instances almost a year

Panayotatou (1913) considered the water of the Nile River in Egypt, in which in contrast to many rivers in other parts of the world no cholera like vibrios could be found as rather unsuitable for the persistence of *V cholerae* As noted before she ascribed this unsuitability (which was only relative in degree as the vibrios were found capable of surviving in raw Nile water for periods ranging from 1 to 13 days as against at least 30 days in boiled or sterilized samples) to the presence of bacteria antagonistic to the cholera vibrios. Gohar & Makkawi (1948) recently finding that when cholera stools were added to crude Nile water the vibrios survived for four days only as against nine days if pure cultures of *V cholerae* were used for such tests, ascribed the difference to an inimical action of the bacterial species normally present in the faeces.

Further observations on the vitality of cholera vibrios in the water of India may be summarized as follows

Jolly (1926) raised an interesting point when drawing attention to observations which showed " the occurrence of a change in the reaction

of Ganges river water from alcaline to acid beginning about April before the onset of the rains, and swinging back from acid to alcaline in October or later " Since the two waves of cholera in Eastern Bengal and Assam coincided with the " neutrality points " twice reached by the pH values of the Ganges water, Jolly suggested that a causal connexion might exist between the two phenomena, the infection dying out " rapidly at times when the reaction of the water is either too acid or too alcaline for the organisms and when the temperature is also unfavourable "

Though it is difficult to share Jolly's belief that an alkaline reaction of the Ganges water might have proved inimical to *V cholerae* one must admit that the presence of an acid reaction of the water might have played a role, in addition to the prevalence of temperatures unsuitable for the survival of the organisms

Khan & Agarwal (1929) comparing the survival of cholera vibrios in unboiled and boiled samples of the Ganges and Jumna water, recorded the following results

Kind of water	*Survival (days)*	
	unboiled	*boiled*
Ganges	1 ±0.62	2 ±0 46
Jumna	1 ±0 62	7 ±1.97
Well water	1 ±0 47	3 ±0 86

Comparing the results which they obtained in the four individual sets of tests they made during a period lasting from February to April Khan & Agarwal noted that the duration of life of *V cholerae* in their samples became shorter hand in hand with an increase in temperature and absolute humidity They recorded in this connexion that at the time of their first experiment the mean temperature was 73°F (approximately 23 C) and the mean average humidity 0 388 whereas the corresponding figures for the third set of tests were 86 F (30°C) and 0 607

In a further study Khan (1930) tried to determine to what cause the supposed vibriocidal action of the Ganges water was due He could find no evidence of the existence of volatile vibriocidal substances, noting in particular that water samples heated under conditions which would have prevented the escape of volatile substances no longer exerted an untoward influence on the survival of *V cholerae* Khan ascribed, therefore the apparent loss of vibriocidal power occurring already when the samples had been heated for half an hour at 55 C before they were used for tests with *V cholerae* to the fact that during the process of heating the bacteria naturally present in the water were killed Thus, instead of competing with the cholera vibrios for the available food material, as they did in raw water they furnished in their dead bodies additional nutritive substances for the vibrios. It served as corollary for this assumption that (*a*) in Ganges water samples, which had been passed through Chamberland filters, the cholera vibrios though persisting longer than in raw water died markedly

more rapidly than in heated water samples, apparently because the filtered water was less rich in food materials than the heated water and (*b*) in the experience of Khan as well as of most other observers sterile distilled water was an unsuitable substrate for the survival of *V cholerae*

D Hérelle Malone & Lahiri (1930) testing various Indian water samples (mainly well water no river water) found that the period of survival of *V cholerae* in these specimens was short, as a rule not exceeding 24 hours, and lasting maximally two to four days They stressed the fact that the life span of these organisms appeared to be much shorter in India where cholera was prevalent, than in Europe, which was usually free from the infection

Studying once more the viability of *V cholerae* in certain waters of India, Lahiri Das & Malik (1939) recorded the following findings

Kind of water	*Raw*		*Candle-filtered*	
	untreated	*autoclaved*	*untreated*	*autoclaved*
Hill spring	1 hour	18 hours	—	—
Calcutta tap-water	18 hours	24 hours	2 days	12 days
Hooghly River	18 hours	3 days	2 days	2 days
Tanks *	up to 72 hours	up to 12 days	7 days	18 days

* With a high salt content and rich in organic matter

Lahiri Das & Malik were inclined to ascribe the considerably longer survival of the cholera vibrios in their autoclaved samples to a break down of suspended organic matters facilitating the nutrition of the cholera vibrios.

It should be noted that further tests with Hooghly river water carried out in 1942 under the auspices of the Indian Research Fund Association, showed somewhat longer periods of survival of *V cholerae* amounting to three to four days in the case of raw water to periods up to three weeks in the case of specimens which had been passed through L_3 Chamberland candles.

In the course of their investigations on the growth and survival of *V cholerae* in water Read et al. (1939) to whose important work attention has already been paid above did not test raw water samples. However they carried out tests with autoclaved samples of water from Calcutta tanks, in which they noted survival periods of the cholera vibrios ranging from about five to over 30 days. A general conclusion reached by these workers was that

"Available figures of analyses of natural waters in the Calcutta area suggest that the requisite conditions for multiplication and survival [of *V cholerae*] as far as salt content and organic matter are concerned are present in most of the natural sources."

Read et al also adduced evidence to show that there was a relationship between the prevalence of cholera and a high monthly average of total solids in the Calcutta waters.

Since the important observations of Read & Pandit (1941) on the persistence of *V cholerae* in the natural waters of rural areas in India are of

epidemiological significance rather than being germane to the subject of bacteriology they will be discussed in a later part of this book

Sea water Observations on the survival of *V cholerae* in sea water seem to have been made first by Nicati & Rietsch (1885) who as quoted by Gotschlich (1903) succeeded in isolating cholera vibrios from the much contaminated sea water of Marseilles France harbour and establishing that these organisms could survive in sterilized sea water for periods up to 81 days

Jacobsen (1910) working with water from the Copenhagen Denmark harbour (salt content varying from 8 9 to 16 per thousand) found that in this substrate cholera vibrios persisted for 7 17 days during August and September up to 47 days in November and December and ascribed this difference to a higher microbial content of the water during warm weather

Comparing the vitality of *V cholerae* in artificially contaminated samples of water from the bay of New York and from the Atlantic Ocean with that in two kinds of tap-water (Brooklyn and Staten Island) Gelarie (1916) recorded the following results

	Survival in days (at room temperature in the dark)	
Kind of water	*unsterilized*	*sterilized*
Bay water	7-47	154-285*
Ocean water	7	285
Tap-water	1 or 3	1 or 18

The organisms were still viable at the end of these observation periods.

Long periods of survival of *V cholerae* in artificially prepared sea water were recorded in 1933 by Yasukawa (22 days at the surface and 30 days at the bottom of a tank) and in 1939 by Venkatraman (at least 74 days survival in 2% salt water) On the other hand Flu (1921) noted a survival of the vibrios in the water of the port of Batavia Java, for four days only while Gohar et al (1948) working with samples from some Egyptian ports found that the vibrios disappeared from raw sea water in about 24 hours A moderately long persistence of the organisms in sea water was noted by some early Japanese observers quoted by Takano and co-authors (1926) thus

Author	*Length of survival of V cholerae in sea water*
Nogami (1902)	Raw sea water 3-4 days at 37°C 9-10 days in the ice-box. Sterile sea water 30-42 days at 37°C, 53-65 days in the ice box
Yano Okazaki & Hiroumi (1904)	At 37°C, 12 days in raw and 83 days in sterile sea water at room temperature 26 and 152 days respectively in a dark room at room temperature, 41 and 209 days respectively in the ice-chest (3 -8°C) 27 and 230 days respectively
Matsuda (1920)	7 10 days in raw sea water not exposed to direct sunlight, depending upon the degree of contamination of the water Exposure to direct sunlight rapidly killed the vibrios.

Remarks

(*a*) According to Yasuhara (1926) whom Takano and colleagues could not quote, cholera vibrios survived during winter for 11 days in the sea water of Katsuura port, for 8 days in the water of the river mouth, for 11 days in the river water

(*b*) Some of these workers, carrying out parallel tests with fresh water found that here also survival of *V cholerae* was considerably longer at lower than at higher temperatures.

Concluding remarks The evidence adduced above makes it clear that the chances of a survival of cholera vibrios in water depend upon an interplay of a number of variable factors such as the temperature and pH of the water its salt content, the amount of organic matters present, and the degree of bacterial contamination. There is little room for doubt that, provided conditions for the subsistence of *V cholerae* do exist, the temperature of the water which in turn depends upon the prevailing season, is one of the main factors perhaps the principal factor determining the length of survival of the organisms.

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Chapter 4

PROBLEMS IN IMMUNOLOGY*

Toxin Production

Dealing with the cholera problem at a conference held in 1884 at Berlin Koch considered this disease essentially as a toxicosis, caused by a " poison " which the causative organisms excreted However the experimental evidence soon procured by some workers was not in favour of the presence of such an exotoxin. Thus Nicati & Rietsch (1884) the pioneers in this field, found the filtrates of young broth cultures of *Vibrio cholerae* incapable of producing signs of toxicosis in dogs However an intoxication, charac terized by vomiting, dyspnoea or general depression, and paralysis of the extremities, could be produced in dogs injected intravenously with the filtrates of cholera cultures a week or more old, and led in some of the animals to death within 12 hours.

Further classical experiments by Cantani (1886) showed that intra peritoneal injection of dogs with p ptonized broth cultures of *V cholerae* which had been sterilized by heating at 100 C, produced in these animals severe though passing, signs of intoxication not dissimilar from those observed in human cholera Subcutaneous injection of dogs with the same material led to less marked signs that of peptone free broth cultures was without effect—a difference due in Cantani s opinion to a more rapid growth as well as a more rapid death of the vibrios in peptonized broth. Generally speaking, this worker ascribed the appearance of the toxic signs he could produce in the above-described manner to the action of an endotoxin of the *V cholerae* which, set free after the death of the organisms, acted in a manner comparable to that of mushroom poisons

The assumption of Cantani that the cholera vibrios, while not secreting an exotoxin, contained a potent endotoxin was confirmed by observations of some other early workers, particularly the profound studies of Pfeiffer (1892, 1894b see also Pfeiffer & Wassermann, 1893) and of Gamaleia (1892a)

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Summarizing the results of his initial investigations, Pfeiffer (1892) stated that

"quite young, aerobically cultivated cholera growths contain a specific poisonous substance which exhibits extremely toxic effects. This primary cholera toxin stands in a very close relationship [*in sehr enger Zusammengehörigkeit*] to the bodies of the organisms, forming perhaps an integral constituent of them The toxic substance undergoes apparently no change if the vibrios are killed with the aid of chloroform thymol or through drying.
"Absolute alcohol concentrated solutions of neutral salts, boiling heat decompose the poisonous substance, leaving behind secondary toxins which exert a similar physiological action but produce an identical effect only in a 10- to 20-fold dose" [Trans]

It should be noted in this connexion that, according to Pfeiffer (1894a) in the case of the primary toxin obtained from chloroform treated cultures of *V cholerae* doses varying from 2.5 mg to 5 mg per 100 g of body weight of the experimental animals were necessary to produce death in intra peritoneally infected guinea pigs

As described by Pfeiffer (1892) the most conspicuous sign of cholera intoxication in these animals was an incessant drop of their body tempera ture which, apt to commence as early as 1½ to 2 hours after the toxin admi nistration, became most marked (as low as 30°C intrarectally) in fatal cases Hand in hand with this temperature drop the animals became prostrated their hind extremities became paralysed, and fibrillary convulsions of the musculature could be observed. If a sufficiently high dose had been given, death usually occurred after 12 16 hours Administration of lesser doses led to a less marked drop of the body temperature (e g. to 34 C) and, though apt to show serious signs of intoxication, the animals became well after 24 hours.

Gamaleia (1892) whose independent work fully confirmed the finding of Pfeiffer came to the conclusion that the labile primary cholera toxin was a "nucleo-albumin" which became converted through exposure to tems peratures above 60 C or to strong chemicals into a more stable "nuclein" What relationship exists between this substance and the "nucleoproteid" of the cholera vibrios prepared by Galeotti (1896) and considered by this worker to be the endotoxin of *V cholerae* is difficult to decide It is impor tant to note in this connexion that comparative tests made by Bürgers (1910) with (*a*) cholera agar cultures treated according to Pfeiffer s methods to obtain the primary endotoxin, and (*b*) growths heated to high tempera tures and consequently supposed to contain only secondary toxins, failed to show as marked differences as had been found in corresponding experi ments by Pfeiffer and Gamaleia

It is of historical interest to note that the statements made by Pfeiffer and Gamaleia met at first with considerable opposition, several workers (enumerated in the summaries of Kolle & Schürmann, 1912, Kolle & Prigge 1928 and Kraus 1929) maintaining that the pathogenic action of *V cholerae* was due not to an endotoxin but to the secretion of a soluble exotoxin

by the living organisms However as convincingly shown by Kolle & Prigge (1928) these claims which as a rule were based upon tests with only one or a few strains—quite often of a rather doubtful nature—deserve no credence Thus, as recently stated by Wilson & Miles (1946) it is now generally accepted that

" the cholera vibrio does not secrete a true soluble exotoxin but that it contains endotoxins which are liberated on the autolysis of the bacilli in culture or on the active disintegration of the bacilli by the cells of the animal body The analogy that it presents with the meningococcus—another organism that readily undergoes autolysis—is very close, though the cholera vibrio is far more toxic "

Recent observations on the toxin of *V cholerae* as far as they fall within the scope of the present disquisition may be summarized as follows

Boivin et al (1934) reported that they had been able to obtain from various Gram-negative bacteria including the *V cholerae* through extraction with trichloracetic acid in the cold an opalescent fluid which gave specific precipitation reactions with immune sera prepared against the organisms in question and which through prolonged boiling with weak acetic acid could be split into (*a*) a nitrogen and phosphorus-containing precipitate, and (*b*) a specific polysaccharide which remained in solution Further studies of the " glucido-lipoid complex " obtainable through extraction with trichloracetic acid led Boivin & Mesrobeanu (1935 1936) to the conclusion that this compound containing the principal part of the endotoxin of the organisms in question as well as their somatic antigen represented their " complete antigen " whereas the specific polysaccharide, because it was incapable of producing antibodies and antitoxins upon injection into rabbits corresponded to a residual antigen or hapten. The two workers maintained in this connexion that, when the organisms had become rough, i e devoid of their specific somatic antigen, they were endowed only with a feeble toxicity due to the bacterial proteins. The latter representing the " acid insoluble " part of the endotoxin were of little importance in determining the toxicity of the smooth organisms, due principally to the acid soluble portion of the endotoxin as characterized above

As summarized by Burrows (1944 see also Burrows et al 1946) the findings of Boivin and his co-workers were confirmed by several subsequent observers such as Raynal Lieou & Feissolle (1939) Dambovicenau & Barber (1940) and also by Gallut (1943) to whose work reference is made below

A method of separating the cholera toxin from the vibrios without the use of chemicals was described by Banerjee (1942) who for this purpose took advantage of the technique of cultivation in Cellophane bags devised by Gildemeister & Neustat (1934) Slightly modifying the procedure of these workers, Banerjee used

"a long cylinder in which 2 cellophane sacs are placed, each tied tightly with string to a tube open at both ends. The length of the tubes are adjusted so that they project through the opening of the cylinder The open ends of the glass tubes and the cylinder are plugged with cotton wool. 200 c.c. of Ramon s medium [¹] is put in the cylinder outside the sacs and 50 c.c. of the same medium is put in the sacs through the glass tube This is then sterilised in the autoclave and dialysis was allowed to proceed overnight at room temperature. In another similar apparatus oil of vaseline is put in the cylinder and also in the sacs to serve as anaerobic culture"

The culture medium in the bags was inoculated *in situ* with one fifth of a slant of a 24-hour-old cholera culture and the apparatus was incubated at 37 C for 18 hours The Cellophane bags were then removed from the cylinders and snipped with scissors so as to empty their contents

As was established in the course of Banerjee s work, equally good growth could be obtained if the Cellophane bags were filled with normal saline instead of Ramon s broth because satisfactory diffusion from the medium surrounding the bags took place

To remove the vibrios from the cultivation fluids high speed centrifugation for 20 minutes was used Then, since the supernatant fluid was not quite free from vibrios a current of air charged with a mixture of toluol and chloroform was passed through it

Testing the toxicity of the fluids thus obtained Banerjee found that —regardless of whether aerobic or anaerobic cultivation had been used—all guinea pigs injected intraperitoneally with amounts of 2 ml died within 24 hours whereas nearly all receiving 1 ml succumbed within 4 days The minimum lethal dose for intravenously infected mice was 0 25 ml

A rapid method for obtaining cholera toxin was described by Bernard & Gallut (1943a) thus

The broth medium used by these two workers was that recommended by Ramon (1933) for the production of highly potent diphtheria toxin.

In 20 ml of this broth to which 5 g of glucose and of sodium acetate had been added per litre, cholera vibrios harvested from three 18-hour-old agar cultures in Roux bottles were suspended, i.e., a quantity corresponding to the weight of 8-10 mg of desiccated organisms per ml. The suspension was then kept at 37°C. To the portions removed from it for the purpose of testing, toluene was added and prolonged centrifugation was then used to obtain a fluid free from vibrios

It was found that toxin began to appear in the suspension after 3 hours and reached a maximum after 4 hours incubation when the lethal dose of the centrifugate was 0.25 ml for a guinea pig weighing 250 g and 0 05 ml for a 15-g mouse respectively upon intraperitoneal administration.

A still more potent toxin could be obtained by using Ramon s broth in quantities of 250-500 ml, centrifuging it after 4 hours incubation at 37° C so as to remove the vibrios, then restoring the original glucose content (5 per 1000) and the original pH (8.0) and

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[¹] As summarized in the *Bulletin of Hygiene* (1933), Ramon's medium was prepared as follows "One litre of water and 10 cc of pure HCl is added to 225 gm. pig s stomach which is allowed to digest at 45°C. for about 2 hours 325 gm. of minced veal is then added and the product is stirred vigorously After 20-22 hours of digestion heat for 20 minutes at 80-100°C. Pass through a bag-filter Adjust to pH 8 with NaOH Heat to 100°C. for 20 minutes Filter through paper Fill out in litre quantities. Autoclave at 108-110°C. for 45 minutes. Add glucose 1.5 to 2 gm. per litre and sodium acetate 5 to 10 gm. per litre. The glucose can be added before sterilisation and the acetate (in the sterile condition) afterwards, or vice-versa the acetate first and glucose afterwards"

again implanting cholera vibrios at the above-mentioned rate. By repeating these operations 10 times, a toxin was obtained, the lethal dose of which for guinea-pigs of 250 g was 0.05 ml. However quite often the toxicity became attenuated, or even altogether disappeared, after the 5th addition of fresh vibrios. Nevertheless, as pointed out by Bernard & Gallut, even without resorting to cumulative procedures it was possible to obtain with their new method within four hours a cholera toxin, the potency of which was at least equal to that of the toxin produced by vibrios cultivated in the usual manner for 7 days in Ramon's broth without glucose.

The two workers added in a second note (1943b) that in the course of cholera toxin preparation according to the above-described method, 99.5% of the vibrios were found to be dead after an incubation of four hours when the pH of the medium had fallen to 5.8. In the opinion of Bernard & Gallut,

"the wholesale [massive] death [of the vibrios] under the influence of a pH of 5.8, due to the fermentation of the glucose, appeared to be the dominant factor in the rapid diffusion of the cholera toxin under the conditions of our experiences." [Trans.]

Carrying out comparative studies with (*a*) the glucolipidic substance obtained from smooth cholera vibrios according to the method of Boivin & Mesrobeanu (1936) and (*b*) the toxin extracted after four hours according to the procedure of Bernard & Gallut (1943a) Gallut (1943) found that

(1) The content in glucolipoids in the toxin prepared according to Bernard & Gallut was higher than that obtainable from the smooth cholera vibrios themselves with the aid of Boivin & Mesrobeanu's method, usually twice as high.

(2) As suggested by a positive biuret reaction, the toxin prepared according to Bernard & Gallut contained besides the glucolipidic complex also a variable quantity of proteins and polypeptides, which could be separated from the fluid containing the non-dialysable glucolipidic complex with the aid of dialysis through highly permeable membranes.

In order to confirm whether the glucolipidic complex of the toxin was different from the glucolipidic substance extracted from the vibrios with trichloracetic acid, Gallut & Grabar (1943b) resorted to comparative precipitin tests with a serum which had been obtained by the immunization of rabbits with the latter substance (see Gallut & Grabar 1943a). The conclusion reached by the two workers on account of these tests was "that at an early stage of its elaboration the toxin contained a more complex antigen which was afterwards split into a more simple glucolipidic compound (like that extracted from the vibrios themselves) and a substance not precipitable with the immune sera."

Further studying the cholera toxin with the aid of ultrafiltration, Gallut & Grabar (1945) confirmed that the toxin consisted of two different substances—namely (*a*) a simpler glucolipidic antigen and (*b*) a toxic substance which, because of small molecular size could pass the ultrafilters. The two workers thus characterized the differences existing between these two substances

	Glucolipidic endotoxin	*Toxic substance in ultrafiltrate*
Dimensions	80-100 mμ	4 mμ
Chemical nature	glucolipoid	probably proteid
Action of immune sera prepared either with the total toxin or with the simpler glucolipidic complex	neutralized	not neutralized
Pathogenic effect	producing congestion	producing hypothermy

More detailed studies of the hypothermy producing component of the cholera toxin by Grabar & Gallut (1945) rendered it likely that contrary to the previous beliefs of these workers the substance, because found to be non precipitable by trichloracetic acid or by sodium tungstate was probably not of a proteid nature The hypothermy producing substance was found to be thermolabile and to possess no antigenic power (see also Gallut & Grabar 1947)

Burrows (1944) used the following methods to isolate the endotoxin of *V cholerae* (*a*) extraction in the cold with M/2 trichloracetic acid followed by dialysation of the neutralized extract through Cellophane (*b*) disintegration of the vibrios by high-speed grinding with sand, followed by centrifugation so as to separate the cellular debris from the toxic opalescent supernatant (*c*) solution in 6M urea (*d*) digestion with pepsin for 3-5 days, followed by removal of the insoluble material through centrifugation (*e*) extraction of lyophilized vibrios with methyl alcohol, ethyl alcohol chloroform or ethyl ether in a Soxhlet apparatus

The addition of 3-5 volumes of ethyl alcohol to the trichloracetic acid extract resulted in the appearance of a flocculent precipitate (found to be a polysaccharide) while the toxin remained in solution When the filtrate from alcoholic precipitation was concentrated by evaporation a yellow oil separated out which, containing most of the endotoxin, had a mouse MLD (minimal lethal dose) of 0 1 ml and appeared to be a mixture of alcohol and lipids, probably similar to the substance isolated from the *V cholerae* with the aid of trichloracetic acid extraction by Raynal Lieou & Feissolle (1939)

While the preparations obtained in the manner just described proved unsatisfactory because of the difficulty of separating the toxic substance from the trichloracetate and the method of direct alcohol extraction of the vibrios proved inefficient, extraction of the dry (lyophilized) vibrios with alcohol or chloroform proved highly satisfactory The crude material thus obtained was yellowish in colour It appeared to consist of a mixture of lipids and contained in the case of alcoholic extracts considerable quantities of inorganic salts Purification of the crude extract could be effected by successive acetone precipitation and resolution in minimal quantities of hot absolute alcohol The white lipid material thus obtained was negative to the Molisch, Millon, and biuret tests, and was found to contain minimal values of 5% nitrogen and 0 7% phosphorus With proper care this

material, which represented about 2% of the dry weight of the vibrios could be persistently prepared with a mouse MLD of 30 µg.

Burrows concluded from these investigations which, as will be discussed later were amplified by important observations not falling within the scope of the present disquisition, that the endotoxin of the cholera vibrio was (1) stable to acids but unstable to alkali (N/10 NaOH at room temperature) (2) readily soluble in methyl and ethyl alcohols, chloroform and ether but not in glycols (3) readily dialysable and (4) " closely related possibly identical with a phospholipid "

In a preliminary note referring to further studies of the cholera endotoxin in Burrow's laboratory Freter (1953) stated that the endotoxin of cholera vibrios grown in 1% glucose peptone water could be partially extracted in trichloracetic acid or pyridine 70%-80% remaining in the cells. While the rapid extraction method of Bernard & Gallut gave similar results, better yields (30%-40%) could be obtained by extracting the vibrios with dilute acid at pH 3 8 for 4 hours. Freter added that

" the soluble toxin so obtained could be purified by coprecipitation with calcium phosphate or carbonate, precipitation with acetone and deionization by treatment with a mixture of anion and cation exchange resins that resulted in precipitation of inactive material "

The purified substance containing 4.5% N and 1 7% P had an LD_{50} of about 0.2 mg

As further found by Freter the residual toxicity from vibrios treated with HCl could be brought into solution by drying with acetone and further extraction at neutral pH The toxic extract thus obtained was not soluble at pH 3 8 and no toxic material could be extracted from the preci pitate at this pH This as well as other differences in chemical and physical behaviour seemed to indicate that the toxin of the cholera vibrio occurred in two different fractions—a conclusion which appears to be analogous to that reached by Gallut & Grabar

Referring in greater detail to the above-mentioned investigations, Freter (1956) characterized the differential properties of the two endotoxin fractions obtained by him as follows

	Purified acid-soluble toxin	*Purified acid-insoluble toxin*
Dilute acid	Soluble	Insoluble
Chloroform-water emulsions	Activity in supernatant	Activity in emulsion
Ion exchange resins	Not adsorbed	Adsorbed
Percentage nitrogen content	4.5-6.0	12 14
Percentage phosphorus content	1 5-2.0	1 0-1.5
Reducing substance — before hydrolysis	0	1.0-1.5
Reducing substance — after hydrolysis	8-10	6-7
Lipid (%)	10	4
Amino-sugars	+	—
Ninhydrin test	—	+
LD_{50} (mg)	0.05-0 10	0.35-0.75

As Freter further established both these fractions were stable to heat (100 C for 10 minutes) and d d not diffuse through Cellophane membranes, hence the acid insoluble moiety of the toxin appeared to be different from the protein like substance described by Gallut & Grabar (1945) The ac d soluble moiety of the toxin on the other hand, appeared to be similar in LD_{50} nitrogen content and solubility to the toxin fraction described by these French workers (see also Bernard & Gallut 1945) and by Burrows (1944) Freter emphasized

"that the extraction of the toxic fractions described above did not require any steps which could not be realized in the infected human or animal body (pH 5 6 or 8.5 breaking of the bacterial cells, use of fresh vibrios, not ageing cultures) Consequently [he con tinued], there is no reason to believe that slightly different environmental conditions, such as might be present during the actual cholera infection could not favor the production of endotoxin fractions with similar degrees of toxicity but with varying chemical and physical properties."

However suggestive though these postulations and the observations referred to above in general are in the opinion of the present authors the bulk of the evidence available in regard to the endotoxin of *V cholerae* coupled with what is known on bacterial endotoxins in general (see summary by Burrows 1951) is more consistent with the hypothesis that the cholera endotoxin occurs as a single substance of sufficient lability to become altered by extraction and purification procedures As a consequence toxicity with an original or altered pharmacological activity might be found associated with a variety of biochemical properties giving the appearance of several toxins and leading to divergent reports in the litera ture on the nature of the cholera endotoxin

Observations on the behaviour of the better known endotoxins of the enteric bacilli especially the dysentery bacilli deserve great attention in this connexion. As summarized by van Heyningen (1950) and by Burrows (1951) the studies of Goebel and his colleagues have shown that toxicity lay in a relatively small basic component of the intact endotoxin of the Flexner dysentery bacillus and that this could be prepared linked with either the polysaccharide or the polypeptide portion of the endotoxin molecule but could not be separated in active form. Such an active moiety in this and other endotoxins was termed "TOX" by van Heyningen (1950) who suggested that the diffusible toxic substance of low molecular weight obtained by Burrows (1944) with the aid of alcohol extraction possibly represented the TOX portion of the cholera endotoxin molecule Whether such a view on the nature of the cholera endotoxin is valid remains for further studies to determine

Besides the observations mentioned above several studies on the toxin of *V cholerae* which deserve attention at the present juncture, have been published by Gallut (1953d 1954a, 1955) and by Gallut & Jude (1955)

Gallut (1953d 1954a) reported that he had been able to study the toxigenicity of 40 cholera strains which had been isolated from 10 severely affected patients in every instance on several occasions and usually four times These growths which were all of the Ogawa type and with one exception, smooth had been lyophilized immediately after their isolation in Calcutta and as a rule had been subcultivated once only To determine their toxicity Gallut resorted to intraperitoneal infection of white mice For each strain at least four groups of these animals were used, receiving respectively 0 2 ml 0 1 ml 0 05 ml and 0 025 ml of cholera toxin produced according to the method of Bernard & Gallut (1943a) described above The LD_{50} for the experimental animals was calculated by the method of Reed & Muench (1938) and the titre of the toxins was determined by ascertaining the LD_{50} values per ml of the various preparations. Gallut found in this manner not only that the toxicity of the different strains varied from patient to patient but also that the growths successively isolated from each sufferer differed While the organisms isolated early in the disease—and, more markedly still, the growths obtained at the end of the attacks—appeared to be less toxic a "hypertoxic" vibrio furnishing from 1 5 to 7.5 times more toxin than the other growths of the same origin could be isolated from each sufferer in the course of the illness, on the average 60 hours after the onset of the attack As Gallut pointed out, this particularly high toxicity of *V cholerae* becoming apparent, as it did at about the middle of the cholera attack, could easily have remained unnoticed, since as a rule examinations were either made soon after admission of the patients to establish the diagnosis or late in the disease or in convalescence in order to ascertain whether the causative organisms were still present in the stools

After following up these investigations with observations on the experimental virulence of *V cholerae* (see below) Gallut & Jude (1955) and Gallut (1955) used the technique described above to study the relation between the toxicity of cholera strains *in vitro* and the temperature of incubation Working at first with an Ogawa strain Gallut & Jude found that

"The toxigenic power of the cholera vibrios varies according to the incubation temperature of the cultures. The most active toxins are obtained when the culture is maintained at 18 to 20°[C], the most feeble at 41 The toxins produced at 37° show an intermediate activity" [Trans.]

The two authors added that

"The cultures developed at 41 which are feebly toxic, regain by passage at 20 merely a partial activity inferior to that of cultures grown at 20° and even at 37° [Trans.]

Reporting upon a continuation of these studies Gallut (1955) stated that (*a*) the results obtained with the above mentioned Ogawa strain had been confirmed through examination of other strains of the same type and (*b*) identical findings had been made with one cholera strain of the Inaba type.

Making similar tests with stock strains, Gallut came to the conclusion that

"Aging of the strains, like incubation at 41 also diminishes the toxigenic power of *V cholerae* yet leaves the organisms with a toxicity which is not altogether negligible" [Trans.]

It was claimed by several observers particularly by Kraus and his co-workers (see summary by Kraus, 1929) more recently also by Takita (1939) that—in contrast to the classical non haemolytic *V cholerae*—the El Tor vibrios produced in addition to an "haemotoxin" (haemolysin), as is generally accepted, also an exotoxin However the existence of such a separate exotoxin distinct from the haemolysin of the El Tor vibrios has been rendered rather doubtful through interesting observations of Pottevin (1913b) mentioned later and of Gohar (1932a) Determining the haemolytic power of the supernatant fluids of El Tor broth cultures which had been centrifuged after incubation for five days and testing at the same time the toxicity of these fluids by intracutaneous injection of rabbits in analogy with the method devised by Kovacs (1932) Gohar established that toxicity appeared to run parellel with the haemolytic power of the fluids He further found that absorption of the fluids with cholesterol suprarenal tissue, or brain tissue i e. with substances rich in lipoids, rendered the fluids atoxic as well as non haemolytic while brain tissue extracted with ether to remove the lipoids as far as possible failed to produce these effects The fluids likewise became atoxic if sheep erythrocytes had been haemolysed in them and it was even found that

"If in a haemolysis experiment the last tubes containing the weakest dilutions and showing slight or no trace of haemolysis are tested for toxicity they are found to be nontoxic"

Gohar concluded from these observations that "the haemolysin and the exotoxin are probably one and the same thing" He admitted that old El Tor strains which had lost a great deal of their toxicity remained haemolytic Presumably however this merely indicated a degradation of the toxin the more so as it remained immunogenic.

Virulence

Dealing in a general manner with the problem of bacterial virulence Wilson & Miles (1946) stated that

"the term *virulent* is sometimes used as though it were completely synonymous with *invasive* but this usage is unjustified by derivation and singularly inconvenient in practice. If rigidly adhered to it would necessitate the exclusion from the class of virulent bacteria of all of those organisms that exert their lethal effect by the production, in localized foci of powerful toxins It is better practice to retain the term *virulent* in its correct sense of poisonous, without any implication as to how the poisonous effect is produced and to apply it to any organism which gives rise to a rapidly fatal infection."

Even apart from the fact that an invasion of the human body by the *V cholerae* does not lead to a generalized infection this broad definition

of the term "virulence" is particularly adequate for the special case of this organism which, though not producing true cholera if administered to experimental animals with the aid of the usual techniques, nevertheless is apt to cause death in these animals as well as in man if introduced in its virulent form even in a small dosage However while it is legitimate therefore to utilize the usual animal experiments, particularly intraperitoneal infection of guinea pigs for an assessment of the virulence of cholera strains, it must be realized that, as far as the experimental animals are concerned, virulence defined as above is an attribute not only of the classical cholera vibrio as is the rule for man, but also of the El Tor vibrios and even some of the cholera-like vibrios

A further but only apparent difficulty is created by the problem of the *relation existing between the virulence and the toxicity of the vibrios* Some of the early workers, considering human cholera attacks the result of a toxicosis and also bearing in mind that general signs identical with those following the introduction of living cholera vibrios could be produced in experimental animals by the administration of killed organisms or even of culture filtrates apparently thought the terms of virulence and toxicity to be interchangeable Dungern (1895) noted in this connexion that, as shown by the observations of Pfeiffer (1894a) freshly isolated cholera vibrios were able to multiply in the peritoneal cavity of guinea pigs if introduced in small quantities whereas organisms grown for prolonged periods on artifical media could do so only if administered in large doses The question arose therefore, whether this was due to a difference in the resistance of freshly isolated and long cultivated cholera vibrios to the bactericidal substances of the animal body or depended upon a difference in the toxicity of the strains in question, rendering them more or less able to counteract the bactericidal substances.

To answer this question, Dungern made comparative tests with (1) a freshly isolated East Prussian cholera strain so virulent that $\frac{1}{8}$ $\frac{1}{4}$ of a loop (0.25-0.5 mg) of 20-hour old agar cultures was lethal for intraperitoneally infected guinea-pigs, and (2) an 8-year old often subcultivated stock culture originally isolated from a cholera patient in Calcutta which, if administered intraperitoneally in large doses (10 mg or 20 mg), produced death from toxaemia with negative bacteriological findings, the introduced vibrios having obviously been killed.

The toxicity of these two strains, tested by intraperitoneal or intravenous injection of guinea-pigs with chloroform- or heat-killed organisms, was almost exactly identical. However while a guinea-pig intravenously injected with 2 mg of living vibrios from the recently-isolated culture showed a rapid drop of the body temperature and died in less than 24 hours, yielding abundant growths of *V cholerae* from the blood, spleen, liver and the haemorrhagic peritoneal exudate, two guinea-pigs, injected with 2 mg of the Calcutta strain, survived, showing a passing slight drop of the temperature (to 35°C) in one instance, some fever (maximum 39°C) in the other Dungern maintained that the dose of 2 mg was below the lethal one in the case of either strain but that obviously the virulent vibrios (East Prussian strain) were able to survive and to multiply

The conclusion reached by Dungern on account of these experiments was "that the virulence of cholera bacilli can be quite independent of their toxicity"

The fundamental facts of cholera virulence have been elucidated through the systematic investigations of Pfeiffer (1892, 1894a 1894b) and Pfeiffer & Wassermann (1893)

Pfeiffer established in the course of his initial work (1892) which, though carried out with a strain found afterwards to be not of a true nature was fully confirmed by subsequent investigations with immunologically identified cholera vibrios, that in the case of living organisms the lethal dose for intraperitoneally injected guinea pigs of about 400g body weight was usually about one loop occasionally as little as ½ loop To kill the animals by subcutaneous infection at least 5 to 10 times higher doses were necessary The dose necessary to cause rapid death in intraperitoneally injected guinea pigs with chloroform or thymol killed cholera cultures was about three times higher than that needed in the case of living organisms—an observation supporting Pfeiffer s assumption that, though in both cases the action of the cholera toxin was the immediate cause of death, in the case of live vibrios introduced in small doses an initial multiplication of the organisms in the peritoneal cavity was an indispensable prerequisite

Pfeiffer & Wassermann (1893) stated similarly that

in the case of intraperitoneal injection of live cultures the excess [*Plus*] of toxic substances which are formed through proliferation of the vibrios in of the peritoneal cavity is most essential and, given a high virulence of the culture, can be a multiple of the amount of toxin transmitted with the originally injected bacterial substance " [Trans.]

In fact, these two workers defined virulence, as far as their investigations were concerned "as the capability of the vibrios in question to multiply in the guinea pig peritoneum"

As stated by Pfeiffer in a subsequent study on the etiology of cholera (1894a) he was able to continue work on the virulence of *V cholerae* with numerous fresh strains isolated mostly in Germany during the recent European cholera manifestations

According to these observations

"the cholera cultures, regardless of whether they had been derived from most severe and rapidly fatal cholera cases or from instances of slight infectious diarrhoea, showed a remarkably uniform behaviour In the case of intraperitoneal infection, the minimum lethal dose was invariably but part of a loop (of a total capacity of 3-4 mg culture material) $\frac{1}{6}$ or $\frac{1}{8}$ of a loop usually sufficing to kill the guinea-pigs. The subcutaneously infected guinea-pigs, on the contrary showed only a feverish reaction lasting a few hours, while pigeons (infected intramuscularly with 1 loop) survived" [Trans.]

Only three of the many cultures tested showed an aberrant behaviour killing guinea pigs infected subcutaneously with ½ 1 loop and occasionally even pigeons Such an extreme virulence of cholera cultures seemed so unusual that Pfeiffer seriously doubted the true nature of these three strains

However as pointed out by Pfeiffer (1894b) the virulence of strains subcultivated for prolonged periods was apt to become abated or even lost. The strain afterwards used by Dungern (see above) in particular had completely lost the ability to subsist in the guinea pig peritoneum, vibrios injected into the peritoneal cavity of normal (i e non immune) animals disappearing within 20-30 minutes without evidence of marked phagocytic activity

Gruber & Wiener (1892) who also studied the virulence of *V cholerae* stressed that, especially in the case of agar cultures only quite young (i.e., 15-30 hours old) growths were fully infectious whereas material from cultures 48 hours old or older caused as a rule only illness of varying severity but no death, or was even altogether inactive That the age of the growths exerted an important influence upon their virulence seemed also indicated by the observation that intraperitoneal injection of guinea pigs with material from the actively growing marginal portions of 48-hour old agar cultures still proved lethal, whereas material from the centre of such growths failed to produce this effect As claimed by Gruber & Wiener the lost virulence of old cholera cultures could be quickly restored through subcultivation

Flügge (1893) and Gotschlich & Weigang (1895) were not in accord with the postulation of Gruber & Wiener that the cholera vibrios were infectious only in the stage of their youthful vigour (*vollste Jugendkraft*) whereas later they lost their virulence without impairment of their capability for saprophytic growth. Paralleling determinations of the number of viable organisms in cholera cultures of different age with virulence tests performed through intraperitoneal infection of guinea pigs Gotschlich & Weigang were able to show that in all instances, regardless of the age of the growths one and the same number of viable cholera vibrios, approximately 200-300 million represented the minimum lethal dose They likewise established that by keeping their cultures at room temperature or in the ice box instead of at 37 C they could not only prolong the viability but also preserve the virulence of the growths, cultures kept at lower or low temperatures for 2 3 days proving as virulent as those in the "full vigour of youth" Gotschlich & Weigang concluded, therefore that

"in one and the same culture the virulence of the individual viable organisms is of a constant size the virulence of the culture is the resultant of the actions of the individual organisms the changes of the virulence taking place in aging cultures are due solely to quantitative differences in the number of viable vibrios, not to qualitative changes taking place in the individual bacilli" [Trans.]

Acceptable though this conclusion remains as far as recently isolated cholera vibrios are concerned, it has to be pointed out that (*a*) as shown already by Pfeiffer (1894b) the virulence of old often subcultivated strains was apt to become lost and (*b*) as recently found, first by Shousha (1923)

and generally accepted, dissociation into the rough state leads to a decrease or loss of the virulence of *V cholerae* due to qualitative changes [1]

The postulate of Pfeiffer (1894a) that no parallelism existed between the virulence of different cholera strains and the severity of the disease they produced in man has been confirmed by most subsequent observers However in contrast to the above noted experiences of Pfeiffer it is now generally accepted that regardless of the character of the manifestations which they produce in man the virulence of different cholera strains is apt to vary within fairly wide limits Discussing this problem Gotschlich & Weigang stated that the unequal virulence of the various strains might be due to differences in the rate of multiplication of the growths in question or to innate racial differences and adduced some evidence suggesting that both these possibilities were of actual importance

As shown by some early workers such as Haffkine (1892) and Gotschlich & Weigang (1895) and confirmed by ample further observations passage through intraperitoneally infected guinea pigs is an effective means to restore or if serially repeated, even to enhance the virulence of cholera strains Gotschlich & Weigang referred in this connexion to one strain, the minimal lethal dose of which was reduced through 7 passages directly from animal to animal from a value of over 2600 million organisms to 900 million Even more spectacular results were recorded by Kabeshima (1918a) in the case of an El Tor strain which had been subjected to passage through a series of 45 guinea pigs

Claims that the virulence of cholera strains could be enhanced by other means, e g. through growth in diluted immune serum (Hamburger 1903) through symbiosis with other bacteria (Puntoni, 1913a) or through short term exposure to a temperature of 48 C (Sulman 1933) seem not to have been supported by further observations The same seems to hold true of the contention of Melnik (1925) that as shown by guinea pig experiments, (*a*) cholera vibrios which had been cultivated on agar became maximally virulent when 16 hours old but rapidly lost their virulence whereas (*b*) growth of the organisms in broth led to a slow increase of the virulence (maximum after 4 days) followed by a gradual decrease

Recently the problem of the virulence of *V cholerae* has been studied once more by Jude & Gallut (1955—see also the preliminary communication of Gallut & Jude 1954) as well as by Husain & Burrows (1956)

The French workers intraperitoneally infecting lots of white mice with saline suspensions of one stock strain and four recently isolated and immediately lyophilized cholera strains found that the virulence of Ogawa strains of *V cholerae* for the white mouse varied according to the incubation temperature of the growths, those grown at 18 C proving most virulent

[1] It is also interesting to note that cholera strains which had become dependent for their growth on streptomycin were, according to Olitzki & Olitzki (1955), avirulent for mice when given intraperitoneally in mucin in doses up to 10^{8} organisms, and for guinea-pigs if suspended in broth in doses up to 4×10^{8} organisms.

and those incubated at 41 5 C proving least so Incubation of the growths at 37 C led to a progressive loss of virulence which became more marked as the number of subcultivations increased and which like the virulence loss of the cultures grown at higher temperatures was but partly reversible Considering these findings and the absence of any differences when the sera raised against strains cultivated at these three temperatures were used for absorption and cross-agglutination tests Jude & Gallut were led to conclude that

"The variations of the virulence under the influence of the temperature of incubation seems to be conditioned solely by variations in the toxigenic power [of the strains]." [Trans.]

In the course of an exhaustive study on the virulence of cholera vibrios for the mouse Husain & Burrows (1956) examined a total of 47 serial isolates of *V cholerae* obtained either from 15 patients who had recovered from the disease or—in four instances—from sufferers who had succumbed to it, in the sixth transplant from the original isolation plates by the conventional LD_{50} method Results indicated that no clear correlation existed between the mouse virulence of the organisms, used in 5 % mucin suspensions for intraperitoneal inoculation of the animals and the virulence of human cholera, as judged by clinical criteria On the contrary it appeared that (*a*) "nonfatal cholera in man can be caused by vibrio strains of mouse virulence as great as that of strains from fatal cases, and also by strains of much less virulence for the mouse" and (*b*) as far as the experiences of the authors went, fatal human cholera was not invariably due to an infection with strains of a high mouse virulence Hence the conclusion reached was "that the mouse LD_{50} criterion of virulence is in fact, quite irrelevant to cholera in man"

However resorting to periodic quantitative examinations of the blood of the infected mice Husain & Burrows were able to classify their strains as follows

(1) Smooth strains of predominantly fatal origin, which increased rapidly in the blood, "possibly as a consequence of an increasing rate of dissemination from the peritoneal focus of infection, multiplication in the blood stream, or failure of defense mechanisms to remove them, or any combination of these factors" However the maximal level of bacteraemia, reached just before deaths began to occur in the lots of mice concerned, remained moderate, possibly because of invasion of other tissues from the blood.

(2) Smooth strains of predominantly non-fatal origin increasing in the blood only at a moderate rate, "possibly because the spillover from the peritoneal cavity does not increase as the infection progresses or because multiplication in the blood is insignificant"

(3) R strains, which appeared to be limited in their ability to spread from the focus of infection in the peritoneal cavity and reached only moderate numbers in the blood and then declined.

Discussing the significance of these and related findings Husain & Burrows stated that

"If fatality in the human infection may be taken as an indicator of the relative virulence of *V. cholerae* for man, the data reported here suggest that such virulent strains of the microorganism are demonstrably more invasive in the mouse. There is no a priori reason for such an association since Asiatic cholera is an extreme example of a true enteric infection, one in which there is little or no invasion of the tissues from the focus of infection in the lumen of the bowel. The greater invasiveness of vibrio strains of fatal origin is perhaps no more than an association with, or a reflection of, other elements of virulence of the microorganisms more nearly related to the pathogenesis of the disease in man."

Be this as it may, the studies of Husain & Burrows lend support to the postulation that, as far as cholera in man is concerned, the virulence of the infection is not merely a function of the toxicity of the causative organisms.

Antigenic Structure

Early observations

The scanty references made to the antigenic structure of *V. cholerae* during the period immediately following the introduction of the agglutination test as a means for the identification of this organism by Gruber & Durham (1896) are of historical interest rather than of actual importance. While, as summarized by Meinicke, Jaffé & Flemming (1906), Gruber & Durham as well as some other early observers assumed that differences in the virulence of the various cholera strains were the cause of their different agglutinability with a given immune serum, a few workers, such as Durham (1901) and Kolle & Gotschlich (1903), postulated that differences in the receptor apparatus of the organisms accounted for the discrepant serological results. This assumption was refuted by Meinicke and co-workers, who declared "that the cholera cultures possess the same receptors in about equal quantities but that the avidity of the single receptors to the antibodies of the cholera serum differs in the various cultures." However, Kraus, Hammerschmidt & Zia (1911) re-asserted that cholera strains were apt to vary in their antigenic structure and that the presence of special agglutinogens was the cause of the discrepant behaviour shown by the strains which had been isolated during the 1908 outbreak in Kamaran, Arabia. Kraus and his co-workers insisted, however, that the different behaviour of these strains could be demonstrated only with immune sera possessing a low titre. Similarly, Ohta (1914) claimed that the action of low titre sera was different from that of sera with a high titre and that with the aid of the former the cholera vibrios could be divided into two types.

Definite proof of the existence of different serological types of *V. cholerae* was adduced through a study of over 200 strains by Kabeshima (1913), to whose observations detailed reference will be made later in this chapter.

Greig (1916), testing 39 more or less haemolytic vibrio strains isolated from Calcutta surface waters, found "that the antigenic character of

and those incubated at 41 5°C proving least so Incubation of the growths at 37°C led to a progressive loss of virulence which became more marked as the number of subcultivations increased and which like the virulence loss of the cultures grown at higher temperatures, was but partly reversible Considering these findings and the absence of any differences when the sera raised against strains cultivated at these three temperatures were used for absorption and cross-agglutination tests Jude & Gallut were led to conclude that

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Discussing the significance of these and related findings, Husain & Burrows stated that

" Our anti-cholera sera obtained by injection of Koch s vibrios heated during 2 hours at 100 C, agglutinated our different strains of cholera vibrios whether they were heated to 100 C or not quite as well as anti-sera of 56 C prepared at the same time As absorption tests confirmed the agglutination experiments we state that all their antigen is thermostable " [Trans.]

Soon afterwards however the fine studies of Balteanu (1926) proved that the conclusion arrived at by Weil & Felix and by Brutsaert was due not to the absence of thermolabile antigens in the *V cholerae* but merely to the difficulty of demonstrating their presence

In the introduction to his paper, Balteanu stated that

" The motile bacillus possesses two distinct kinds of agglutinable substances one labile, the other stable when subjected to 100 C. or to dilute acid or absolute alcohol The antisera contain special agglutinins corresponding to each kind of antigen The labile factor and its agglutinins are responsible for agglutination in large, loose flocculi the stable factor and its agglutinins for agglutination in small compact granules. The isolated flagella react as if composed entirely of labile material. The agglutinins for the labile and stable antigens are in the inverse order susceptible to heat those for the labile antigens resist a temperature of 70 C. for 20 minutes those for the stable are inactive after such treatment. The non motile races of normally flagellate organisms contain only the stable antigen and their properties are limited thereby they agglutinate only in small granules and their agglutinins are destroyed at 70 C. This is the standard scheme of serological properties which has been kept in view in studying the antigenic complex of *V cholerae* "

In his initial studies of this problem Balteanu used, besides the poly valent immune serum of the Lister Institute London, sera of his own, prepared by immunization of rabbits with suspensions of a cholera culture which had been heated before injection for 30 minutes at 58°C and for 2 hours at 100 C repectively Carrying out agglutination and absorption tests with the aid of these sera, he was able to prove that the four cholera strains studied by him possessed H as well as O antigens However there was sometimes a poor contrast between the floccular (H) and the granular (O) forms of reaction—obviously due to the fact that in the case of the monoflagellate *V cholerae* the ratio of heat labile constituents was low as compared to that met with in the *Proteus* and paratyphoid groups How ever Balteanu was able to overcome this impasse by shaking and then centrifuging the suspensions of cholera agar cultures so as first to liberate the flagella and then to separate them from the bodies of the vibrios which were mostly thrown down during centrifugation He found that the clear fluid obtained in this manner in which only very few bodies of vibrios but abundant flagellar material were present

(1) agglutinates in typical flocculi with an ordinary anticholera serum made with an emulsion of whole cholera vibrios killed at 58°C. It no longer functions after being heated at 100°C

"(2) When inoculated into rabbits it induces the production of a serum which makes flocculent clumps with a flagellar suspension and reacts almost exclusively with the flagellar labile constituents of an ordinary emulsion of the vibrios."

vibrios isolated from water is different from that of the standard cholera vibrio as known to bacteriologists" As shown by cross-agglutination tests with sera obtained by immunizing rabbits with these water vibrios, 32 of them fell into 6 serological groups while 7 remained ungrouped. Included among the latter were two strains of apparently atypically behaving cholera vibrios

Observations by Mackie & Storer (1918) afterwards confirmed and amplified by Mackie (1922) showed that "paracholera" vibrios isolated in Egypt from patients with choleraic disease were likewise serologically distinct from true cholera vibrios

That the last mentioned and also some earlier observers even though they worked with agglutinating sera which were not fully specific according to modern standards obtained surprisingly clear-cut results was—as Gardner & Venkatraman (1935b) aptly pointed out—due to the fact that

"The agglutination method used by Greig, Mackie, etc., involving a low temperature (37°C) and a relatively short period of incubation (2 hours), reveals in general only 0 agglutination, and so enables a distinction to be made between the various 0 subgroups."

The validity of this contention is proved by the conclusions of Mackie (1922) who maintained that

"[a] By direct agglutination tests, using plain saline emulsions and incubating at 37°C for 2 hours, the paracholera vibrios are distinctly differentiated from *V cholerae*

"[b] *V cholerae* antiserum exhibits apparent co-agglutination under certain conditions towards *paracholerae* A and certain similar types this effect develops more slowly than the agglutination of the homologous organism and is of lesser degree and of lower end-titre it is most markedly elicited when formol-broth emulsions are used and the tubes are incubated first at 55 C"

Heat stable (O) and heat-labile (H) antigens

While the observations recorded above indicated that the cholera vibrio possessed an antigenic structure different from that of the cholera like vibrios and also that the latter fell into numerous immunological groups they failed to explain the nature of these differences and—more generally speaking—furnished beyond some rather vague speculations no clue as to the character of the antigenic make up of the organisms.

An investigation of the latter problem was rendered possible through the classical studies of Weil & Felix (1920) on the antigenic make up of typhoid and paratyphoid bacilli even though the two workers concluded from some preliminary tests that in contrast to these bacterial species the *V cholerae* had no double antigenic structure Observations suggesting that this organism possessed thermolabile as well as thermostable antigens were recorded in 1921 by Miyake and by Watanabe but these findings, published in Japanese medical journals, attracted no attention. Brutsaert (1924) supported the tentative conclusion reached by Weil & Felix, stating that

to the group of vibrios sharing with *V cholerae* both its antigens but differing in their being haemolytic "

Besides the so-called El Tor strain mentioned above two of the cholera like vibrios were agglutinated by H+O cholera serum in the living state but not after they had been heated for two hours at 100 C. Abdoosh concluded therefore that the heat stable antigen of *V cholerae* was " sharply specific " for this organism and the El Tor vibrios in the strict sense How ever some cholera like vibrios, though differing in their somatic antigens were related to the cholera vibrios by virtue of their heat labile antigen

Gohar (1932b) examining 45 cholera and cholera like strains main tained on the contrary that the relationship of the latter to *V cholerae* " may refer to either the flagellar or somatic antigen " In his opinion therefore absorption tests were necessary in addition to agglutination tests in view of the fact that the cholera like strains possessing the same antigens as *V cholerae* were found incapable of absorbing all the antibodies from a cholera serum.

However the importance of O-agglutination for the laboratory diagnosis of cholera was again emphasized by Taylor (1934) and White (1934a) in reports rendered to the Office International d Hygiène Publique in response to an inquiry into the preparation of standard agglutinating sera for this diagnostic work. The observations recorded in this connexion by Taylor indicated that smooth cholera strains could be best distinguished with the aid of heated suspensions of the organisms while White maintained that

" The identification of *V cholerae* depends in fact on the O agglutinins and it seems necessary to envisage the opportunity to make the diagnostic tests with a pure O serum i.e. with a serum raised against a vaccine heated to 100°C or naturally devoid of flagellar antigen."

The validity of this proposal was fully confirmed by Gardner & Venka traman (1935b) whose publication may be considered the charter of the present knowledge on the antigenic structure of cholera and cholera like vibrios

Gardner & Venkatraman used for their comprehensive studies 101 cholera and cholera-like strains which according to their biochemical reactions could be divided into (*a*) " typical " vibrios, i.e., those producing acid without gas in glucose, maltose, mannite and saccharose, giving the cholera red reaction, and not fermenting dulcite (*b*) " atypical " vibrios, found to be divergent in one or more of these characters but showing a general similarity to the typical vibrios and (*c*) " non fermenting " vibrios, markedly different from the previous groups by failure to acidify any of the above mentioned carbohydrates and also by an inability to produce the cholera red reaction or to liquefy gelatin.

To test these strains serologically Gardner & Venkatraman worked with H-O and O suspensions, and with H+O as well as with pure O sera.

Having established that, in contrast to what was the case in the *Salmonella* group formol did not inhibit the O agglutinability of vibrio suspensions, Gardner & Venkatraman prepared their H-O suspensions by the addition of 0.2 / formol and 0.2 / chloroform to 24-hour-old veal-broth cultures (pH 8 0) O suspensions were obtained by placing

Balteanu s general conclusions which have been confirmed by all subsequent observers were that in *V cholerae*

"(1) There are two series of antigenic substances which may be termed stable (O) and labile (H') respectively (Somatic and Flagellar antigens according to the terminology of Th. Smith) Of these the stable elements resist a temperature of 100 C. for a considerable period while the labile elements are thereby destroyed.

(2) The agglutination of the stable constituents by themselves (as illustrated by the agglutination of steamed suspensions) takes a purely granular form the reaction of the isolated labile elements (as shown by the agglutination of living emulsions and flagellar suspensions by ordinary immune sera previously absorbed with steamed cultures) is definitely flocculent

"(3) The combined reaction of the labile and stable constituents in living emulsions to immune sera made with ordinary cultures leads to a mixed flocculent and granular type of clumping except at the upper limit of the titre where the looser and more fluffy type is dominant."

It is of interest to add that the immotile opaque variant of *V cholerae* met with by Balteanu (discussed in the section on "Cultural variation" in Chapter 3 page 126) behaved in the main like an "O" form, since no heat-labile "H"-agglutinable substances could be demonstrated, while the heat-stable somatic, "O" factor was conspicuous However serum produced with this variant contained H agglutinins.

A further most important contribution to the knowledge on the antigenic structure of *V cholerae* was made by Shousha (1931a 1931b) through a study of two strains which had been isolated at the El Tor quarantine camp from pilgrims not suffering from choleraic disease and which were found to be agglutinable with one of the two available cholera-immune sera Shousha was able to establish that the receptors which these two strains had in common with true cholera vibrios were heat labile (flagellar) "group" receptors, whereas the somatic (O) receptors of the two suspect vibrios were quite different from the somatic antigen of *V cholerae* Shousha stressed therefore the importance of using in cholera laboratory work adequately heated suspensions of the cultures to be tested or of preparing sera by immunizing animals with heated suspensions of the organisms so as to produce sera free from H agglutinins He also recommended with great reason that subcultures of the strains used for this purpose be issued together with the sera for the purpose of control tests

Abdoosh (1932) examining 22 strains of true cholera vibrios six strains labelled El Tor as well as three "paracholera" strains and 24 other cholera like vibrios, found that none of the cholera like vibrios possessed the same somatic antigen as *V cholerae* However three of the strains labelled El Tor had the same thermostable and also the same thermolabile antigen as the classical non haemolytic cholera vibrios, while one was agglutinated by cholera H+O serum, but not by cholera O serum. Abdoosh advised in this connexion that it was essential "to confine the term El Tor

As summarized by Takano Ohtsubo & Inouye (1926), Kabeshima based his observations on an examination of 195 cholera strains recently isolated in Japan and Formosa and of 19 stock strains from European laboratories. He found that according to their serological reactions these strains could be divided into a "typical" and an "atypical" group each of which agglutinated at high titre with homologous immune serum but weakly with sera raised against strains of the opposite type The presence of these two types could be confirmed with the aid of agglutinin absorption complement-fixation and bactericidal tests. Kabeshima postulated that the different behaviour of the two types was due to the presence in each of two different antigens, a principal one responsible for the reactions with homologous sera, and an accessory one, reacting with the heterologous sera. The strains isolated during the 1912 cholera epidemic in Japan were typical in character while those derived in the same year from Formosa belonged to the atypical group

The existence of two serological types of *V cholerae* was soon confirmed by several other Japanese observers (see summaries by Nobechi 1923 and Burrows et al 1946) Pratt (1925) also described two serological types, the presence of which, though not invariably revealed by agglutination could always be demonstrated by cross absorption tests

Nobechi (1923 see also Nobechi 1933) proved the existence of a third serological type of *V cholerae* standing between Kabeshima s two groups which are now usually designated as the Inaba and Ogawa types As summarized by Nobechi (1923) the strains of his new "middle" type (now often designated as the Hikojima type)

"are agglutinated by the sera of the two other types, capable to differentiate the strains of the two types from each other to the same titre with corresponding strains and the middle type sera, with no exception, agglutinate all strains of the other types as well as of the middle type almost uniformly high. From the result of the agglutinin absorption test, the middle type strains studied by the author are assumed to be provided with the common antigen X, and the original type specific A, at the same time also with the varied (i.e. atypical) type specific B though the development of the last is incomplete."

In his second paper (1933) Nobechi characterized the antigenic structure of the three types thus

Type	*Specific fraction*	*Common fraction*
Original (Kabeshima s "typical" group)	A	X
Intermediate (Hikojima)	AB	X
Variant (Kabeshima s "atypical" group)	BC	X

Aoki & Oshiro (1934) claimed that the occurrence of specific thermostable antigens and/or of unspecific partly thermolabile antigens accounted for the differentiation of *V cholerae* into three types According to this concept, the vibrios of the Inaba type had only specific receptors those of the Ogawa group only unspecific receptors while both kinds of antigen were present in the intermediate (Hikojima) type However as pointed out by Burrows et al (1946) it is difficult to correlate this assumption with

dense harvestings from 24-hour-old agar cultures in saline for two hours into boiling water Such prolonged heating was found to be indispensable to destroy completely the antigenic action of the H component, but is was noted that a few minutes exposure to 95 100°C was sufficient to remove the H agglutinability of the suspensions. Alcohol treatment and growth of the vibrios on phenol agar were also tried to destroy the H component, but gave no satisfactory results.

H+O sera were prepared by immunizing rabbits with formolized unheated suspensions, pure O sera were manufactured with saline suspensions which, as noted above, had been exposed to boiling temperature for 2 hours.

Confirming and amplifying the observations of previous workers Gardner & Venkatraman were able to establish that

(1) As shown by cross-agglutination tests with unheated suspensions and O sera, the "cholera group" of vibrios i e the above mentioned categories of "typical" and "atypical" vibrios possessed a diversity of specific O antigens, six of which, being met with in more than one strain, rendered it possible to classify most, though not all, of the organisms of the group into six subgroups All the standard stock strains of *V cholerae* examined and also the majority of races isolated from patients with typical epidemic cholera fell into one group called "I" and the same held true of the majority of the haemolytic vibrios tested, which were thus identified as El Tor vibrios in the strict sense

(2) As demonstrated by the action of O sera on boiled suspensions, there existed in addition to the specific O antigens a common O antigen the nature of which could not be definitely elucidated. The evidence regarding a possible extension of the non specific O agglutination to vibrios outside the cholera group was also not conclusive but Gardner & Venkatraman drew in this connexion attention to White s observation (1934b) on "Q" antigens which will be discussed later in this chapter

(3) As shown by agglutination tests with formolized unheated broth suspensions and H-O sera, the vibrios of the cholera group possessed a common H antigen.

Gardner & Venkatraman urged on account of their experiences that for the identification of *V cholerae* a standard subgroup "I O" serum should be used in conjunction with tests for haemolysis.

Before dealing with further investigations regarding the antigenic structure of *V cholerae* in general attention has to be devoted to the evidence on the existence of serological races of this organism as well as to the antigens present in dissociated vibrios.

Serological races

It is the merit of Japanese observers and especially of Kabeshima (1913 see also Kabeshima, 1918b) to have definitely established the existence of serological races of *V cholerae*

been grown in specific immune serum (and which as became later clear, had thus become rough) showed spontaneous agglutination when suspended in normal saline This phenomenon was further studied by Kabeshima who according to Takano and colleagues (1926) established already in 1913 that

"When the cholera vibrio is cultivated in bouillon containing homologous serum, the organism becomes inagglutinable but it acquires spontaneous agglutinability This is due to the loss of specific receptors, and at the same time new receptors which are common to many strains are formed."

In a further publication (1918c), which was available to the present writer in the original Kabeshima noted that spontaneous agglutination in 0.9% saline was shown not only by strains subcultivated repeatedly in broth containing homologous immune serum but also by 8 out of 19 old stock strains of *V cholerae* However while these 8 strains remained capable of absorbing cholera agglutinins and also remained antigenic, those which had become spontaneously agglutinable in normal saline or inagglutinable with specific serum through growth in the latter, had lost their antigenicity as well as the property of agglutinin absorption—apparently because they had lost their specific receptors Kabeshima also stated that the spontaneously agglutinating strains yielded homogenous suspensions if instead of 0 9% saline, a 0.2% solution was used.

Shousha (1923) as well as Goyle & Gupta (1932) again studying the phenomenon of spontaneous agglutination of *V cholerae* with a full knowledge of bacterial dissociation, confirmed the presence of profound differences shown by smooth and rough strains respectively in agglutination and agglutinin absorption tests but did not correlate these divergent reactions with changes in the receptor apparatus of the organisms However the latter problem received full attention in the studies of Yang & White and of White which have been referred to in part in the preceding chapter

As was noted there and as was also stated by White (1935a) in an article on "The serological grouping of rough vibrios" the serological specificity of the different vibrios depended on their smooth antigens With roughening these differences tended to disappear so that forms which were quite distinct in the S state fell into larger R agglutination groups Transition into the ρ form led even to the disappearance of this group specificity of the rough vibrios so that, as White (1935) put it, "the serology of the ρ vibrio variant is overwhelmingly generalized"

In a further publication dealing with the O receptor complex of *V cholerae* and its antibodies White (1937c) reported on observations he had made when immunizing rabbits with polysaccharide fractions isolated from smooth cholera vibrios These fractions were found to be actively antigenic but the resulting sera showed a varying content of type and group-specific agglutinins similar to that obtained in serum manufacture with whole vibrios Besides being distinct by the range of their (type or

what is known in regard to the H and O antigens of the cholera vibrio In fact the investigations of the workers quoted below leave no room for doubt that differences in the O receptor apparatus are solely responsible for the occurrence of the serological races of *V cholerae*

Scholtens (1933a, 1933b, 1934 1936a, 1936b) stated in this connexion that the cholera vibrios fell immunologically into two groups, about two thirds of the strains possessing only an "A" antigen, the remainder also an additional antigen "B" Both these antigens met with also in the El Tor vibrios were found to be thermostable Identical conclusions were reached by Heiberg (1936) as far as the *V cholerae* was concerned

Gardner & Venkatraman stated in a preliminary communication on their above-described investigations (1935a) that they had been able to confirm through agglutination and absorption tests the existence of "ori ginal" and "variant" types not only in cholera strains from Japan but also in races from India, China, and elsewhere as well as in El Tor strains in the strict sense. They added that

"The reality of the third or middle type is not yet fully confirmed, though some of our experiments indicate that certain of the Japanese races labelled middle type possess, as they are supposed to, both the characteristic antigens of the original and variant types. Contrary to the belief of Inouye & Kakihara (1925), these characteristic antigens are of the heat-stable or O type. They are subsidiary or additional to the main heat-stable antigen that distinguishes them all from vibrios belonging to the other sub-divisions of the cholera group"

Whether these variants of *V cholerae* were stable or fluctuating, was in the opinion of Gardner & Venkatraman still undecided. The claims made by some of the Japanese workers that they had succeeded in transmut ing strains of the variant type into the middle type through growth in immune serum seemed not well substantiated, the less so because according to Gardner & Venkatraman (1935b) in some of the recorded instances at least such transitions had been concomitant with roughening.

In spite of these uncertainties there was however not the least doubt that at least two serologically distinct races of *V cholerae* existed and Gardner & Venkatraman (1935b) urged therefore, that the standard O sera used for cholera diagnosis should contain the subsidiary as well as the main agglutinins of the O subgroup I

Further reference to the serological subgroups of *V cholerae* will be made when dealing below with recent studies on the O antigens of this organism.

R and ρ antigens

That cholera vibrios which have undergone dissociation are apt to react peculiarly in serological tests seems to have been suggested first by attempts made by Hamburger (1903) to increase the virulence of these organisms he noted in the course of this work that vibrios which had

earlier workers—there existed in the cholera vibrios an alcohol soluble antigen comparable to the Q antigen previously isolated by him from salmonellae The total Q fraction produced from agar grown vibrios through alcohol extraction could be divided into (1) a soluble part (Q_1) which could be separated off by treatment of the total fraction with alkalized water and (2) an insoluble Q_2 component which could be precipitated from the residue of the total fraction with the aid of hydrochloric acid

Making further studies of these Q proteins of *V cholerae*, White (1935b) was led to believe that the total Q fraction was identical with the "acid soluble A substance" isolated, with the aid of extraction methods similar to those used by him by Linton and his co-workers (see Linton & Mitra 1934 Linton Mitra & Seal 1935 Linton, Shrivastava & Mitra, 1935)

The immunological properties of the Q antigens were characterized by White (1935b) thus

"Vibrios heated at 100°C. in saline suspension agglutinate in a generalised manner and often to a high titre with the antisera of the Q proteins of the cholera vibrio. The antibodies concerned are not inactivated by the carbohydrate fraction of *V cholerae* Occasional strains of vibrio react similarly in the living state with these Q (cholera) agglutinins. The antiserum of the Q_2 substance of S (smooth) *V cholerae* seems to possess agglutinating properties additional to those of anti-Q_1 and anti-Q_2 sera, rather more specific and possibly related to the carbohydrate receptors. There is reason to believe that the Q proteins are true constituents of the living vibrio and are not serological artefacts due to reagents and heat. It seems that these substances and their antibodies are important contributors to the non-specific O agglutination of vibrios recently discussed by Gardner & Venkatraman."

Heat-labile somatic protein antigen (HLSP) As described by White (1940b) it was possible to extract from chloroform treated young vibrio cultures with the aid of saline "a heat-coagulating antigen common to all known variant forms and seemingly derived from the deeper somatic substance" While accordingly this substance took no evident part in vibrio agglutination, it showed "extremely wide cross precipitation reactions throughout, but not overstepping, the vibrio group"

Heat stable somatic protein antigen (HSSP) Using hot saline solutions for the extraction of chloroform treated vibrio cultures White (1940e) was also able to extract a heat stable somatic protein antigen which, like the HLSP appeared to belong to the deeply situated substances of the vibrios

Anti-HSSP sera (prepared with the aid of rough and ρ strains to avoid an influence of the smooth antigen) gave intense precipitation reactions with extracts of R and ρ strains of cholera and many other vibrios and also with hot saline extracts of the smooth variants of the strains However the anti HSSP sera "did not react visibly with any of the serologically active vibrio fractions with the exception of $C\gamma$ (the polysaccharide fraction brought into solution on proteolytic digestion of R and ρ vibrios)" (White 1936a)

group) specificity the receptor groups of the smooth vibrio polysaccharides were found to be partly alkali labile and partly resistant to alkali. Immunizing a group of rabbits with a given polysaccharide now one then another of these various receptor groups was found to play a dominant role in the stimulation of antibodies As pointed out by White this uncertainty in agglutinin response was bound to complicate attempts to standardize cholera laboratory diagnosis by issuing standard antigens for serum manufacture in local laboratories

Summing up further experiences regarding the rough and ρ antigens of *V cholerae* White (1940d) stated that

"It would seem that the major component in the somatic agglutination of R and ρ vibrios is a heat-stable antigen which, though it perhaps contains protein, is at least considerably resistant to proteolytic digestion This component carries, with certain common receptors most obviously displayed in the reaction to ρ antiserum, the differential receptors of the variants and includes the polysaccharide C β or C δ It is possibly to be regarded as the R or ρ antigen. But the somatic agglutinating apparatus of the variants seems to present other antigenic components, probably in the main common in quality to the R and ρ forms Since they appear to be totally inactivated by proteolytic enzymes, they are probably of a protein nature. Possibly they are combined with the proteolysis-resisting component in a single complex."

In White's opinion the R and ρ agglutinating antigens furnished the "skeletal system" of the cholera vibrio and, being less hydrophile than the smooth antigen, they indirectly conditioned the spontaneous agglutinability of the R and ρ variants by "failing to counteract the hydrophobe tendency of the surface lipoids"

"*Rugose*" *antigen*

Dealing with the immunological properties of the rugose variants of *V cholerae* White (1940a) stated that such races possessed a special antigen which proved to be resistant to heating in neutral solution at 100°C. Sera produced with this antigen reacted not only with the rugose variants of the O subgroup I of Gardner & Venkatraman but also agglutinated rugose races of certain vibrios belonging to other subgroups As White pointed out, the reactions obtained with such heterologous strains "disclose a flaw actual if in practice unimportant in the doctrine of the serological specificity of the heat-stable agglutinogens of O group I vibrios"

From rugose S R, and ρ growths of cholera and El Tor vibrios a common non protein but carbohydrate-containing haptene could be isolated This substance which was found to be absent or inconspicuous in non rugose strains reacted strongly and characteristically with sera prepared from whole rugose vibrios

Other additional somatic antigens

Q antigens In a short preliminary note published in 1934 White stated that—notwithstanding the discrepant results recorded by some

existed within the O subgroup I namely, type A into which 11 of the 50 cholera strains tested fell type AB characteristic of the Ogawa strains type AC, to which the Inaba vibrios belonged and finally type ABC inferred to correspond to the Hikojima type

Since with the exception of the group-specific antigen A which was met with exclusively in *V cholerae* and the El Tor strains in the strict sense, the major antigens were also found in vibrios not belonging to O subgroup I Burrows and his co-workers urged that the identification of vibrios falling into this class "should be based on agglutination with monospecific A anti serum"

Findings confirming those of Burrows et al. were recorded by Gallut (1949a, 1949b) who examined

(*a*) 49 authentic cholera strains, including 35 isolated during the 1947 epidemic in Egypt

(*b*) 12 El Tor strains in the strict sense partly those obtained during the 1938 Celebes outbreak

(*c*) 21 cholera like strains 13 of which were of human origin and 8 isolated from water

As summarized by Gallut the percentage incidence of the 13 antigenic factors in cholera and true El Tor vibrios on the one hand and in the cholera like vibrios examined by him on the other was as follows

Antigenic factors	*Cholera vibrios*	*Cholera-like vibrios*
A	100	0
B	32	47
C	95	14
D	45	19
E	47	9
F	26	0
G & J	1 6	19
H & M	3	4
I & K	18	9
L	47	28

Considering that, in contrast to the factor A, which had been met with only in true cholera and El Tor vibrios the factors B and C, responsible respectively for the type-specificity of Ogawa and Inaba vibrios and jointly for that of the Hikojima type were also present in the cholera like vibrios Gallut urged the use of monospecific anti O sera A for the laboratory diagnosis of cholera He also recommended that, in order to avoid co-agglutinations due to the presence of factors D or E, sera specific for the factors B and C be used for type differentiation In regard to the selection of suitable strains for vaccine manufacture he maintained that

"It is true that the solution so far adopted of preparing vaccine from strains isolated during the epidemic against which control measures are being taken is generally satisfactory but the objection to it is that it is entirely lacking in precision. If the overriding

Thus, as White (1940e) summarized

"There have now been separated from the vibrio bodies (1) a heat labile protein antigen (H.L.S.P.), (2) a heat-stable protein antigen (H.S.S.P.) possibly associated with a haptene Cy2, (3) an alcohol soluble Q protein fraction and (4) the differential agglutinating S, R and ρ antigens with their respective polysaccharide haptenes Cα, Cβ and Cδ. Another haptene Cy1 is probably also of somatic origin, while yet another the rugose haptene has been derived from the intercellular secretion of rugose cultures. A method has been given for separating vibrio flagella. Antibodies for all these components occur or may occur in the sera of rabbits immunised with living cultures of *V. cholerae*. It is certain that vibrio cultures contain other separable serologically active and antigenic constituents and it is by no means unlikely that some of the fractions already described will prove to be mixtures."

Recent observations on the O antigens

The necessity of using, according to the recommendation of Gardner & Venkatraman (1935b) O sera for the laboratory diagnosis of cholera was fully endorsed by large-scale investigations carried out in India and recorded by Taylor (1937 1938 1941)

Experiences identical with those in India were gained by Russo (1938) who recommended repeated (4-6) subcultivations of suitable cholera strains on agar containing 0.5% lithium chloride to obtain growths free from flagella for the preparation of pure O sera. Cultivation of the vibrios on alcohol-containing media or the use of heated suspensions were in Russo's experience less suitable to obtain H free antigens for serum manufacture while growth of the organisms on phenol-containing media was altogether unsuitable for this purpose. He concluded from tests with 58 strains of cholera and cholera like vibrios that with the aid of Inaba O serum it was possible to differentiate Inaba strains from Ogawa and El Tor strains as well as from the non agglutinable vibrios of Finkler Prior and Deneke

An important study based upon an examination of 50 cholera strains 9 true El Tor strains and 11 strains of cholera like vibrios falling into Gardner & Venkatraman's O subgroups II VI was made by Burrows et al (1946). They subjected, for this purpose a group of representative cholera strains to a complete analysis of their heat-stable and heat labile antigens by reciprocal absorption tests and studied at the same time the other vibrio strains with the aid of agglutination with monospecific immune sera. Verifying the tentative O antigenic formulae thus arrived at by absorption tests with known antigens, Burrows and his co-workers found the vibrio O antigens to consist of 13 components five of which were considered as major antigens. One of the latter designated A, was found only in vibrios of the O subgroup I and was, therefore regarded as the group-specific antigen. Antigen B found in 13 out of 20 Ogawa strains but in no Inaba strain, and antigen C met with in all of the 25 Inaba strains examined as well as in two Ogawa strains were considered to be type-specific. The other major O antigens showed no association with the type-specific antigens or with one another. It thus appeared that four immunological types

This has not been confirmed by using the type representative material supplied to us by Burrows and by Gallut

"Of several thousand strains of V cholerae tested by us with cholera O serum (containing group-specific *plus* type-specific agglutinins) not one strain has yet been encountered which subsequently did not agglutinate with type-specific serum, either Ogawa or Inaba Type A serum supplied by Gallut has been found by us to be a non-differential serum containing cholera group-specific A *plus* type-specific C agglutinins It is the same type of diagnostic reagent as is normally used for the preliminary identification of V cholerae and is in no way superior in its diagnostic properties to the non-differential serum used at present in India.

"Antisera raised against so-called A type vibrios—Burrows and Gallut types—were tested against strains of cholera vibrios including freshly isolated and old laboratory cultures. Not a single strain showed positive agglutination. In the light of our experience in India we are of opinion that the existence of a new type of cholera vibrio—containing antigen A only—has not been established nor have we been able to confirm the presence of cholera type-specific B and C antigens in non-cholera vibrios."

In agreement with these observations Venkatraman (1953) recorded in the 1952 report of the Indian Council of Medical Research that an examination of 84 cholera strains including 49 of the Ogawa type and 31 of the Inaba type besides 4 which had become rough failed to show any culture possessing only the group-specific O antigen He added that one of the rabbits which had been immunized with an Ogawa strain yielded a serum containing only type-specific Ogawa O agglutinin being thus completely deficient in the group-specific factor

In contrast to the above mentioned observations Wahba (1951) re examining the 1947 Egyptian strains formerly tested by Gallut (1949) but excluding those which showed abnormal features (i e loss of agglutinability spontaneous agglutination positive results with Millon s reagent, or thermolability) confirmed the multiplicity of the antigenic factors demonstrated in *V cholerae* by Burrows et al and by Gallut. It is noteworthy however, that Wahba found the antigenic formulae of the strains examined by him "not completely stable" He stated in particular that (*a*) the C factor was apt to disappear rapidly in aging cultures and was not demonstrable in formolized suspensions (*b*) the factors D and E, which had been found to be absent in a number of the strains in 1948 were now present, while the L factor, previously demonstrated in several of the strains had become absent. It was also noted that, while the results of agglutination tests became manifest after four hours as far as the major antigenic factors A E were concerned, agglutination of the minor factors took place more slowly becoming manifest only on the following morning.

Evaluating the results obtained by Wahba it must be kept in mind that he worked exclusively with old strains. Thus, as pointed out with great reason by the reviewer of Wahba s article in the *Tropical Diseases Bulletin* his paper "does not appear to help in clearing up the point at issue, which could best be settled by the examination of freshly isolated strains of *V cholerae*"

necessity of having a completely polyvalent vaccine in stock seems acceptable, that is, a vaccine comprising the 13 O factors, it would, however seem logical to take into account the antigenic composition of the vibrios responsible either for endemic cases or a specified epidemic. Only the complete analysis of a sufficient number of strains can furnish this indispensable information " [Trans.]

The validity of Gallut s recommendations for vaccine manufacture was denied by Sokhey & Habbu (1950c) who (*a*) compared the mouse protective power of vaccines prepared with some of Gallut s strains possessing in part a complicated antigenic structure with that of two vaccines manufactured from Haffkine Institute strains which had a quite simple antigenic structure and (*b*) correlated these findings with determinations of the virulence of the strains in question for white mice The conclusion reached was that the protective power of a cholera vaccine depended not upon the complexity of the antigenic structure of the strains used for their manufacture but upon the virulence of the strains chosen Sokhey & Habbu suggested in this connexion

" that the complexity of the antigenic structure observed by Gallut might be due to the degeneration of the strains from age, because they were found to have no virulence to white mice"

Kauffmann (1950) and Singh & Ahuja (1950) approaching the problem with the aid of serological methods were also unable to confirm the results of Burrows and of Gallut

The main conclusion reached by Kauffmann after an examination of 41 strains sent to him by Gallut as well as of six additional cholera strains was that " the occurrence of new types or variants within the O group I that were claimed to be characterized by the antigens D E, F G H I, J K, L, and M could not be demonstrated " However while noting this statement the present writer finds it impossible to share Kauffmann s opinion that " technical errors in the planning and estimation of the serologic examination " accounted for the apparent occurrence of these antigens.

Kauffmann considered a polyvalent O serum, prepared with the aid of Inaba as well as Ogawa strains, to be the most suitable for the identification of *V cholerae* and recommended for the differentiation of the two types of strains a serum obtained by absorption of a polyvalent or an Ogawa serum by an Inaba strain He also advocated the manufacture of polyvalent cholera vaccines with the aid of Inaba and Ogawa strains without giving attention to the serological subtypes as Gallut had urged.

Singh & Ahuja (1950) thus summarized the experiences gained through a serological and biochemical investigation of (*a*) 96 cholera, El Tor and cholera like strains of their own and (*b*) 49 strains put at their disposal by Burrows and by Gallut

" We are sceptical of the claim of Burrows et al. (*loc cit*) that a new type of cholera vibrio has been discovered, namely one containing cholera group-specific antigen only

It is interesting to add that disagreement with the postulations just quoted has quite recently been expressed by Bhaskaran & Gorrill (1957), who insisted that

"experiments with antisera carried out in the cold confirmed that growth of the culture subsequent to the addition of specific antiserum was not necessary for the isolation of the Inaba mutants from Ogawa cultures."

This observation the two authors continued made it very probable

"that these mutants were already present in the parent culture and agglutination by antiserum only facilitated isolation of the mutant in the supernatant fluid The rate of appearance of these mutants was of the order of 1 in 10^9 cell divisions. The inability to demonstrate directly the presence of the mutants in Ogawa cultures was probably due to this low mutation rate, which would require the examination of a similarly large number of colonies for a chance isolation of the mutant The failure to isolate Ogawa mutants from Inaba cultures with the aid of selective antiserum may have been the result of still lower reverse mutation rates."

However Bhaskaran & Gorrill admitted that they had been unable to obtain "unequivocal evidence for antigenic type variation in *Vibrio cholerae* as a result of mutation"

The views on the antigenic structure of the serological types of *V cholerae* expressed by Joya (1950) do not seem significant, because in the opinion of this worker thermolabile antigens played a more important role in the serological differentiation of Inaba and Hikojima strains than the thermostable antigens This is not in accord with the generally accepted doctrine.

In the opinion of Kauffmann (1950) Hikojima and Ogawa strains, because they proved identical in cross-absorption tests, had to be considered one common type He proposed therefore, that a distinction should be made merely between two types of *V cholerae* namely the Inaba type (A C) and the Ogawa Hikojima type with the antigenic formula A B (C) Kauffmann maintained in this connexion that the C antigen though present in small amounts in the Ogawa vibrios, was not well developed in strains grown at 37 C, so that these were incapable of completely absorbing Inaba sera However if the strains were grown at 20 C, they completely or almost completely absorbed Inaba sera In Kauffmann s opinion this was the case because at 20 C the B antigen of the Ogawa strains developed less abundantly and was thus incapable of inhibiting the development or "disponibility" of the C antigen

It is important to note that Gallut (1953b) studying two cholera and two El Tor strains with the aid of single-cell cultivation, came to the conclusion that the Hikojima type even though it showed sometimes a tendency to change into the Ogawa type was a valid race of *V cholerae* Since Gallut was unable to find C antigen in Ogawa strains even if these had been cultivated at 20 C, he rejected Kauffmann s proposal to classify these and the Hikojima strains in a common group as the latter worker had proposed

Recent observations on the serological races of V cholerae

As noted before, Burrows et al (1946) agreeing with the views, but changing the symbols adopted by White (1937c) stated that the antigens B and C of the cholera vibrio were type-specific, while the antigen A was group-specific The antigenic structure of the three serological types of *V cholerae* was therefore, as follows

Type	*Antigenic structure according to* *White*	*Burrows et al*
Inaba	A X	A C
Hikojima	A B X	A B C
Ogawa	B X	A B

The classification proposed by Burrows et al was also adopted by Gallut (1949a, 1949b) and by Kauffmann (1950) to whose postulations reference will be made below

Kabeshima (1918b) had claimed that he had succeeded in transmuting his " variant " (Ogawa) strain into the " original " (Inaba) type by cultivation *in homologous serum and also by inoculation into the gall bladder* of rabbits Whether he also observed transmutations in the reverse direction seems uncertain In analogy with Kabeshima s experiences, Nobechi (1923) was able to transmute through cultivation in homologous serum two " variant " strains into the " middle " (Hikojima) type It is of great interest that observations recorded by Shrivastava & White in 1947 lent support to these early statements of Kabeshima and Nobechi

Shrivastava & White recorded that they had been able

(*a*) to obtain in the case of 10 cholera and 3 El Tor strains of the Ogawa type through cultivation in Ogawa monospecific serum races which were indistinguishable from Inaba type strains and

(*b*) to change, with the aid of monospecific Inaba serum, 4 out of 8 cholera strains which though predominantly Inaba like in serological reactions possessed also an Ogawa factor into the Ogawa type.

It proved impossible, however to produce serological changes other than roughening in 5 cholera and 3 El Tor strains of the strict Inaba type through cultivation in homologous monospecific serum.

Discussing the significance of these observations Shrivastava & White tentatively postulated

" That the Ogawa serological complex represents the known acme of elaboration of the specific somatic antigen of *V cholerae*

" That this antigen is subject to degradation presumably by failure of the organism to synthesize certain chemical groupings.

" That this change is expressed serologically as a positive modification in the detail of antigen and not merely as a factorial loss.

" That from this debased Inaba antigen there is no easy return to the Ogawa state by a revival of lost synthetic power the only escape from the interference of specific antibodies being in the rough change, i.e. entire failure to synthesize the specific complex with resultant unmasking of the R antigen."

It is interesting to add that disagreement with the postulations just quoted has quite recently been expressed by Bhaskaran & Gorrill (1957) who insisted that

"experiments with antisera carried out in the cold confirmed that growth of the culture subsequent to the addition of specific antiserum was not necessary for the isolation of the Inaba mutants from Ogawa cultures"

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It is important to note that Gallut (1953b) studying two cholera and two El Tor strains with the aid of single-cell cultivation, came to the conclusion that the Hikojima type even though it showed sometimes a tendency to change into the Ogawa type was a valid race of *V cholerae* Since Gallut was unable to find C antigen in Ogawa strains even if these had been cultivated at 20 C, he rejected Kauffmann's proposal to classify these and the Hikojima strains in a common group as the latter worker had proposed

As shown by these researches the problem of the somatic antigens of *V cholerae* is still far from being fully solved It is, therefore, not surprising to find that within recent years the optimistic attitude adopted in this respect immediately after the publication of Gardner & Venkatraman s basic observations has been replaced not rarely by one of doubt. Thus, as stated by White in a report rendered in 1948 many experienced cholera workers in India " for one reason or another cling to the view that vibrios other than *V cholerae* may from time to time contribute to cholera "

The validity of this view which implies that the system of cholera *laboratory diagnosis adopted on the basis of Gardner & Venkatraman s* findings on the somatic antigens of *V cholerae* is not sufficiently comprehen sive, was thoroughly discussed during joint meetings of the Cholera Advisory Committee of the Indian Council of Medical Research and the WHO Expert Committee on Cholera (1952) held at New Delhi in 1951 Though the speaker introducing this subject went so far as to consider Gardner & Venkatraman s characterization of the *V cholerae* merely " a precon-ceived notion " in the opinion of the WHO experts,

" the gaps still existing in the knowledge on this subject did not detract from the practical value of the tests adopted for the laboratory diagnosis of the infection. Endorsing this opinion, the committee reached the conclusion that the present definition of the cholera vibrio though incomplete, was sufficient for practical purposes." [Page 6].

Acceptable though this conclusion remains there is a most vital need for further research on the *V cholerae* O antigen This ought to include (1) studies on the antigenic stability of the organism under varied conditions of culture and storage (2) further inquiry into the O antigen complex, especially as regards concealed and blocking antigens and (3) the applica tion of both old and new information to the study of the O antigen complex of vibrio strains of precisely known history and with a minimal number of transplants intervening between isolation and study

Recent experiences on the H antigens

Vassiliadis (1936a) found that treatment of cholera vibrio suspensions with chloroform (1 5 ml per 10 ml of suspension) while leading to a con siderable reduction of the O-agglutinability of the organisms, markedly increased their H agglutinability More than that, some strains of cholera like vibrios, which were not agglutinable with H + O cholera immune serum in their original state showed considerable H-agglutinability after they had been treated with chloroform. As stated by Vassiliadis in a second paper (1936b), this difference was probably due to a removal of lipoids inhibiting H-agglutination by the chloroform. The reduction of O-agglutinability through the action of this reagent was presumably due to the dissolution of lipoids necessary to bring about agglutination.

A method for isolating the flagellar fraction of vibrios was described by White (1940c) who recommended using for this purpose R or ρ cultures on account of their freedom from the smooth specific antigen

The procedure was started by adding chloroform to a dense saline suspension of the vibrios and then stirring so as to separate the flagella from the bacterial bodies. The extract obtained by centrifugation of the chloroform-treated growths after dispersal in saline was treated with an equal volume of saturated ammonium sulfate solution. After 24 hours contact with the precipitant the flagella could be collected and washed with the aid of brisk centrifugation

The preparations obtained in this manner proved suitable in dilution for H agglutination tests, while dense suspensions could be used for the absorption of flagellar agglutinins Injection of the preparations into rabbits stimulated the production of apparently pure flagellar agglutinins

Following up the work of Gardner & Venkatraman (1935) who had shown that vibrios possessing different specific O antigens had the same H antigen Taylor Pandit & Read (1937) tested 558 strains of cholera like vibrios with different sera including an Inaba H + O serum and a serum manufactured with a chemically not fully defined but mainly protein-containing extract of Inaba vibrios The interesting fact was established that, while these two sera agglutinated, besides *V cholerae* strains also many of the cholera like strains this held true only of those giving a cholera red reaction.

Ahuja & Singh (1939) making further studies on 219 vibrio strains which were not agglutinated with specific cholera O serum (subgroup I of Gardner & Venkatraman) found that 35 5% of these cholera like strains possessed H antigens partially or completely identical with the H antigen of *V cholerae* As shown by cross-absorption tests carried out with 10 of these strains and pure H sera (prepared by absorption of sera raised against living suspensions of these strains with massive doses of heat killed cultures of the homologous vibrios) the cholera like vibrios agglutinable with cholera H + O serum apparently fell into three groups, namely,

(1) those possessing an H antigen identical with that of *V cholerae*

(2) strains, the major portion of whose H antigen was identical with that of the cholera vibrios and

(3) strains possessing besides a major individual H fraction a minor one identical with the H antigen of *V cholerae*

The cholera-like strains which were inagglutinable with cholera H + O serum, possessed mainly individual H antigens, though some showed a partial H relationship among themselves

A detailed analysis of the H antigens of *V cholerae* was made by Burrows et al (1946) through reciprocal absorption tests carried out on a representative group of 10 cholera strains with the aid of H + O sera which had been absorbed with their homologous O antigens. The H antigenic structure

of these strains was found to be of a complexity similar to that of the O antigens, but only ten components of the H antigen could be detected, one of which was common to all of the strains Apparently no correlation existed between the variant distribution of the individual H and O antigens respectively

A further study carried out with agglutination tests only on a larger group of vibrios, including, besides cholera and El Tor strains, strains of cholera like organisms, indicated "an apparently random distribution of the H antigens in both cholera and non-cholera vibrios"

Kauffmann (1950) stated that he had been unable to confirm the existence of subtypes of the H antigen

Specially prepared antigens

Basu Chaudhury & Basu (1940) stated that they had obtained a thermostable antigen by (*a*) immersing a Cellophane or collodion bag filled with sterile normal saline in a growing culture of *V cholerae* in peptone solution, and (*b*) filtering the contents of the bag after five days incubation through a Chamberland L_3 candle

The diffusate obtained in this manner contained carbohydrate substances but practically no protein Injected into rabbits, it gave rise to specific agglutinins and precipitins and also protected these animals against lethal doses of *V cholerae*

According to a report published in 1947 Feigina Kuzin & Shapiro obtained through tryptic digestion of cholera vibrios an antigenic complex which however was far less active than the glucido-lipoid antigen of Boivin & Mesrobeanu. Injected into rabbits, the tryptic digest stimulated the appearance of agglutinins but not of precipitins. Hydrolysis of the digest, the antigenicity of which seemed to be due to the presence of peptides led to the separation of nitrogen-containing substances and the loss of antigenic power

Chemical constitution of the antigens

An early attempt to extract the antigens of *V cholerae* with the aid of alcohol was made by Levaditi & Mutermilch (1908) The residue of their extracts obtained through centrifugation and evaporation of the supernatant, proved to be antigenic both in complement fixation tests and in rabbit experiments, and conferred active immunity to guinea pigs. It was apparently thermostable

Since the validity of these findings was doubted by Prausnitz (1911) a further and thorough study of this matter was made by Landsteiner & Levine (1926, 1927)

These two workers obtained by extraction of saline washed cholera vibrios with hot 75% alcohol and further extraction with ether and hot absolute alcohol a solution,

the sediment of which, separated off after cooling (a) reacted in high dilution (1/500 000) in precipitin tests with cholera immune serum, and (b) acted as an antigen when injected into rabbits.

While this crude substance gave both protein and carbohydrate reactions the white powder obtained through purification with alcohol and other reagents was almost protein-free and no more antigenic, but continued to give precipitin reactions up to the above-mentioned titre and positive carbohydrate tests.

In Landsteiner & Levine s opinion, these findings were compatible with the assumption that the crude extracts contained an antigenic complex consisting of protein and a specifically precipitable but non-immunizing complex carbohydrate substance, which probably belonged to the class of " residue " (residual) antigens

Investigations to demonstrate the presence of such residual antigens (haptens) in cholera like as well as in cholera vibrios were made by Jermol jewa & Bujanowskaya (1930) They extracted for this purpose the washings of 24-hour-old agar cultures after digestion with caustic potash with acetic acid and alcohol The substances thus obtained were protein free but gave reactions proving the presence of carbohydrates The extracts gave precipitin reactions with cholera immune serum but were not antigenic when injected into rabbits, unless gelatin or normal pig-serum had been administered simultaneously

Linton (1932) extracted a carbohydrate-like fraction from cholera and cholera like vibrios with the aid of the following technique

"The organisms were sown on Roux bottles and incubated for 48 hours. The growth was then washed off in normal saline, and the solution brought to an acidity of N/20 with glacial acetic acid The bacterial mass was boiled on a sandbath under a reflux condenser until coagulation occurred. The coagulated mass was allowed to cool and then run several times through a Sharples supercentrifuge until a semi opaque brownish solution was obtained. This solution was precipitated with three volumes of 90 % alcohol and placed in the icebox overnight. The heavy precipitate was pipetted off separated from the alcohol as completely as possible by centrifuging, and taken up in 200 or 300 cc. of water Insoluble matter was discarded and the solution again precipitated with alcohol. As before, the precipitate was freed from any remaining insoluble matter and dissolved in 100 cc. of water where it formed a clear brown-tinged solution, with a faint but unmistakable biuret reaction. It was strongly acidified with glacial acetic acid, and boiled. After cooling, the dark brown flocculum which had appeared was centrifuged off and the supernatant fluid, which was now biuret negative, was precipitated with three volumes of alcohol. The precipitate was dried weighed and dissolved in approximately N/20 NaOH to make a 1 % solution "

As Linton added, the solutions thus obtained, while giving negative biuret, Millon s, and xanthoproteic reactions proved positive with Molisch s reagent even at extremely high dilution and, after boiling with dilute acids, were found capable of reducing Fehling s reagent. In cross-precipitation tests the carbohydrate fractions obtained with the aid of the method described above from cholera like as well as from cholera vibrios gave positive results not only with their homologous immune sera but with all

sera tested which comprised besides five raised against *V cholerae* one manufactured with a water vibrio Thus, as Linton put it, the carbohydrate fractions, "if not identical are at least closely related in the agglutinating and non-agglutinating vibrios" (i e., in cholera and cholera like vibrios) The carbohydrate fractions obtained in an identical manner from typhoid and dysentery (Flexner) strains failed to react with any of the six above mentioned sera

The results of further studies on the immunochemistry of the vibrio group by Linton and his co-workers which have already received preliminary attention in the preceding chapter will be dealt with later on.

As noted before, Boivin and his collaborators obtained with the aid of trichloracetic acid, from cholera vibrios as well as from other species of Gram negative bacteria, extracts stated by these workers to represent the "total antigens" of the organisms in question According to Boivin & Mesrobeanu (1935) the substances in question corresponded chemically to a complex combination of specific polysaccharides with fatty acids Exposure of the complete antigens to heating in a weakly acid medium led to the separation of an insoluble portion containing the fatty acids from the polysaccharides which remained in solution. The latter which represented the residual antigens of the organisms could be solidified by precipitation with alcohol or acetone. If redissolved the residual antigens produced solutions which, in contrast to those made from the total antigens, were non-opalescent and weakly dialysable as well as non-antigenic.

On account of the rather fragile nature of the complete antigens, it was possible to split off the specific polysaccharides without trichloracetic extraction directly from the intact organisms by the use of "brutal" methods, such as heating of the bacteria in an acid medium. The same result could be produced by the action of the "diastases" of the organisms

Dambovic eanu & Barber (1940), carrying out chemical analyses of the trichloracetic acid extracts of five cholera strains, confirmed that the complete antigen of *V cholerae* was a glucido-lipoid complex which contained amino-nitrogen and phosphorus. The extracts did not give a biuret reaction and also failed to reduce Fehling s solution, but gave a feebly positive Molisch reaction.

Reviewing the experiences of Boivin and his co-workers as well as of subsequent observers in regard to the trichloracetic acid extraction of *V cholerae* Burrows et al (1946) insisted that none of these workers

"demonstrated either the biochemical homogeneity of these preparations or their postulated identity with the O antigen of the vibrios by cross absorption experiments, nor have the preparations been subjected to immunological analysis"

Burrows and co-workers also laid stress upon the fact that the purified substances obtained by Burrows (1944) with the aid of organic solvents, which were found to consist of phospholipid and additional nitrogenous material gave a negative Molisch reaction.

It has to be added that Linton et al (1938) recorded that they had separated from a cholera strain which had been isolated in the early stage of an outbreak a glucolipid fraction They deduced from this observation that the presence of such a complex might be characteristic of an epidemic type of the organism Apparently however no further observations confirming this assumption have been made

Attention has been drawn in the third chapter to the observations of Linton and his co-workers (see Linton 1940 1942), who were able

(a) by racemization with dilute alkali solutions to demonstrate the presence of two types of protein [1] in the vibrios, the first of which was usually present in *V cholerae* and

(b) also to show the existence of three types of vibrio polysaccharides, most cholera strains being found to possess those of type I (galactose and an aldobionic acid consisting of galactose and glycuronic acid) but a considerable minority showing type II polysaccharides, in which arabinose instead of galactose was found to be combined with an aldobionic acid of the same composition as in the type I polysaccharides.

As maintained by Burrows et al (1946) and also admitted by Shrivastava, one of Linton s principal co-workers, in a 1951 summary the relation of these polysaccharide types to the H and O antigenic structure of the cholera vibrios and to their differentiation in serological races is not clear It is important to note in this connexion that as summarized by Shrivastava (1951) the polysaccharide fractions initially isolated by Linton and his colleagues were found to be serologically inactive and that those prepared later by Shrivastava & Seal (1937) and by Linton Shrivastava & Seal (1938) and Linton et al (1938) though giving precipitin reactions with suitable sera raised against intact vibrios were found to possess no antigenic properties thus falling into the category of haptens

As will be gathered from statements made above, in the course of his immunological investigations White was also able to make observations on the chemical character of the various vibrio antigens demonstrated by him. The following supplementary statements have to be made in this connexion.

(1) *Non protein carbohydrate containing specific substances*

As stated by White (1936b see also 1936a)

"The protein-free polysaccharide specific substance was prepared in a suitable manner by digesting vibrios washed in alcohol and boiled in saline with 1/ papain at pH 5.5 for 7 to 8 hours at 90°C., centrifuging the mixture and precipitating the active substance from the supernatant with alcohol extracting the active material from this precipitate with a saturated aqueous solution of picric acid and then reprecipitating it with alcohol. Picric acid was removed by reprecipitation with alcohol."

As mentioned already White (1937c) found these preparations actively antigenic

[1] These proteins have been further studied by Mitra (1938) who, though finding marked differences in the respective structure of their molecules, admitted that it is impossible with the present data to say whether proteins I and II represent two different entities or whether they are mixtures of several proteins

(2) *Q antigens*

Describing the chemical properties of the protein Q antigens White (1934b) stated that both the Q_1 and the Q_2 antigens gave a positive biuret test, the former antigen reacting less intensely than the Q_2 Both precipitated with Millon s solution, the colour of the precipitate turning to pink or red at room temperature In contrast to Q_2, the Q_1 antigen was soluble in dilute hydrochloric acid. Both antigens proved to be readily soluble in alcohol in the presence of HCl The solvent action of acetic acid was considerably less marked.

(3) *Other special antigens*

Basic chemical reactions shown by the other special vibrio antigens which White (1940b 1940c 1940e) described, may be tabulated thus

Antigen	*Biuret test*	*Millon test*	*Molisch test*
Heat labile somatic protein antigen	intensely positive	intensely positive	definitely positive
Heat-stable somatic protein antigen	strongly positive	strongly positive	strongly positive
Flagellar antigen	intensely positive	imperfect	weakly positive

Note The rugose hapten isolated by White (1940a) gave a negative biuret reaction but an intensely positive Molisch reaction.

Important as these and the previously discussed related observations of White are they merely characterize the chemical composition of the vibrio antigens in a general manner In fact as stated by White (1937c) it was impossible to decide whether his specific carbohydrate-containing antigen was strictly a polysaccharide or a polysaccharide-containing complex of the type described by Boivin and collaborators and also by other workers.

Shrivastava, Singh & Ahuja (1948) tried, for further studies on the immunochemistry of *V cholerae* in addition to the above-described method of White (1936b) the following two methods

(*a*) " The centrifugate of the growth for 72 hours in papain-digested mutton broth is concentrated *in vacuo* at a temperature of 40°C to 45°C. and the concentrate worked for the isolation of polysaccharides [Shrivastava & Seal, 1937]. Protein is removed from the precipitate by shaking it with chloroform and butyl and amyl alcohol."

(*b*) " Phenol method [Palmer & Gerlough, 1940] in which the acetone-dried growth of the bacteria is treated with 90 per cent phenol. This dissolves away the protein and liberates the polysaccharides."

The final product obtained in bulk from an Inaba strain of *V cholerae* with the aid of the last mentioned method, which was found most suitable proved soluble in distilled water and 0 85% saline biuret negative, and Molisch-positive in a dilution of 1 100 000 The nitrogen and acetyl group contents were 7 7% and 2.1% respectively The substance reacted

up to a titre of 1 200 000 with immune sera prepared against both Inaba and Ogawa subtypes of *V cholerae* and, administered subcutaneously to white mice in two doses of 0 2 ml each at weekly intervals conferred to the animals thus treated a high degree of immunity against intraperitoneal infection with mucinized suspensions of *V cholerae* (Inaba subtype)

Singh et al reported in 1950 upon further studies of the *V cholerae* polysaccharides isolated through phenol treatment of acetone-dried growths and subsequent precipitation with 95% alcohol It was possible to obtain with the aid of this method fractions of high antigenicity from Ogawa as well as from Inaba subtype strains As was established in the course of this work, polysaccharide fractions which precipitated at high titre with cholera immune serum did not necessarily confer a high degree of protection to mice Another interesting finding was that intravenous injection of a polysaccharide complex isolated from an Inaba strain into guinea pigs which had been passively immunized with monospecific or non-differential cholera immune serum did not produce signs of anaphylaxis

In contrast to the observations recorded above Sato et al (1950) found that the polysaccharide fractions isolated by them from Inaba and Ogawa strains of *V cholerae* were non antigenic Possibly however this was the result of de acetylation due to the alkali treatment which had been used for extraction

Purified polysaccharide free protein fractions, which were also tested, took in the opinion of Sato et al " a prominent part in the type specific antigenicity in complement fixation tests and agglutinin absorption tests " These are rather surprising results needing confirmation.

Krejci Sweeney & Jennings (1949) reported that they had separated with the aid of electrophoresis from Inaba and Ogawa cholera strains three main constituents of which two (" X " and " B ") showed antigenic activity It appeared that the heat labile antigens were associated with the X constituents the heat stable O subgroup I antigen with the B constituents These were found to contain polysaccharides in combination with proteins The X constituents appeared to consist mainly of proteins or of lipids

A further exhaustive study of the fractions of *V cholerae* separable with the aid of electrophoresis has been made by Burrows (1957) who used for this purpose 32 of the strains previously examined by Husain & Burrows (1956) with the results summarized above The technique used by Burrows was briefly as follows

Suspensions of the strains, which had been grown for 18 hours on thionine-glycerol agar were filtered through gauze and then heated in the water-bath at boiling temperature for 1 hour The heat-stable bacterial material thus obtained was next treated for 40 minutes in a Mickle apparatus with about ¼ volume of glass beads so as to ensure as completely as possible a rupture of the cell walls and a liberation of the soluble contents. After the supernatant had been decanted, the beads were washed three times with distilled water and the resulting fluids were pooled with the supernatant obtained first. The pooled material was filtered through paper centrifuged at high speed and the yellowish,

slightly opalescent supernatant, which represented the intracellular substance of the organisms, was decanted and dried from the frozen state This material was found to contain about 30 / protein, 15 / polysaccharide and 15 / ash.

The cell wall substance, which had been separated from the intracellular material in the manner described above, was washed four times in distilled water then resuspended and treated in an oscillator for 1 hour Afterwards the resulting highly turbid suspension was clarified by high-speed centrifugation, and the supernatant was decanted and dried from the frozen state to be available for testing. It was found that this material contained about 30 / protein and 60 / polysaccharide, but only negligible amounts of ash.

The main results of a study of the electrophoretic mobility of the protein and polysaccharide components of both the cell wall and intracellular fractions obtained were that

" O strains of fatal origin were characterized by the presence of relatively larger amounts of faster moving intracellular polysaccharide and cell wall protein "

and that

" The relative proportions of some of the mobility fractions changed in an apparently systematic way in serial isolates and this was taken to represent an in vivo variation in the microorganisms."

Hence, as Burrows put it, *V cholerae* appeared to be " heterogeneous within the limits of its accepted characterization " It will be important to see how far these changes are correlated to differences in the virulence and toxicity of the organisms

Serological Reactions

Early observations

Profound studies on cholera immunity led Pfeiffer (1895a) to the recommendation of a serological method for the differential identification of *V cholerae* He thus described the technique of this test which is now known under the name of " Pfeiffer's reaction "

" As a rule I use cholera serum, the titre [1] of which is at least 0 001 and take of it for each test 0.01 i.e., ten times the minimal effective dose. One loop of the culture to be tested is mixed with 1 ml broth and the above-mentioned serum dose, and injected intraperitoneally into young guinea pigs of 200-300 g The syringe used for this purpose is provided with a blunt canula. The resistant corium is split with the aid of scissors and the blunt end of the canula penetrates thus quite easily into the peritoneal cavity After 20 minutes I remove with the aid of glass capillaries droplets of the peritoneal contents for examination in hanging drop and stained preparations. If after that time still numerous well-preserved and motile vibrios are present in the peritoneal cavity the reaction is negative and cholera bacteria are, therefore, absent. If on the contrary after 20 minutes in the exudate the injected comma bacilli are found to be changed into granula, among which only quite few and immotile vibrios are noted, there are two

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[1] Pfeiffer (1895) designated as titre of cholera-immune serum that minimal quantity of serum which, if injected together with the cholera dose in 1 ml broth into the peritoneum of young guinea-pigs of 200 g, just suffices to lyse 2 mg of the normal virus within 1 hour

As normal virus he designated cholera cultures possessing virulence sufficient to kill guinea pigs within 24 hours, if injected intraperitoneally in doses of 1/5-1/10 loop (0 4-0.2 mg) of a 20-hour-old agar culture.

possibilities (1) the test culture is devoid of pathogenic properties and thus rapidly destroyed even in normal animals, or (2) true cholera vibrios are present which are lysed by the specific bactericidal substances (positive reaction)" [Trans.]

To decide this issue a control guinea pig was used which received intraperitoneally 1 loop of the culture to be tested + 0 01 ml normal serum in 1 ml of broth. If droplets of this mixture removed from the peritoneal cavity after 20 minutes showed the presence of viable organisms, the diagnosis of cholera was confirmed If on the contrary, the organisms in the exudate of the control animal had disappeared, the result of the test was doubtful in so far as avirulent cholera vibrios as well as cholera like vibrios were apt to be affected by the normal serum Pfeiffer recommended testing under these circumstances the antigenicity of the culture in question through immunization of guinea pigs and stated that it had been possible to confirm in this indirect manner the true nature of an old avirulent Calcutta strain of *V cholerae* Pfeiffer admitted that thus the results of his test were bound to be more reliable the higher the virulence of the cultures under examination was, but added that "as a rule it is absolutely impossible not to come to a decision" In his opinion, an application of the above-described test was indicated only in the case of atypically behaving strains from stools and of water vibrios whereas the hitherto adopted methods sufficed to arrive at a reliable diagnosis in most instances

As had been noted already Gruber & Durham introduced in 1896 the expedient and therefore generally applicable method of agglutination for the laboratory diagnosis of cholera.[1] Achard & Bensaude claimed in the following year that advantage could also be taken of this method by testing the sera of cholera patients with known cholera cultures

Kraus (1897) noted that, as had been previously shown in the case of the typhoid bacillus, cholera vibrios remained agglutinable with specific serum after they had been killed by heating at 56 C. He also made the important observation that the addition of specific immune sera to germ free filtrates of cholera broth cultures led to the formation of precipitates. As proved by controls with normal sera as well as with sera raised against other bacteria, this was like agglutination, a strictly specific test. Since identical reactions could be produced with the juice obtained by exposure of a mixture of cholera vibrios and glass dust to a pressure of 300 atmospheres, the precipitinogens appeared to form part of the bodies of the organisms instead of being excreted by them.

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[1] Bordet had already noted in 1895 that, in contrast to normal rabbit serum, the sera of rabbits immunized against cholera first immobilized and then promptly agglomerated *V cholerae* and that, unlike the bactericidal power this property was not lost when the sera were heated to 55°-60°C. Nevertheless, as summarized by Fitzgerald & Fraser (1928), Gruber & Durham for the first time described the agglutination reaction as a separate and distinct characteristic of immune sera This statement is also valid as far as the observations recorded in 1896 by Pfeiffer & Vagedes are concerned, because these two workers considered the phenomenon of agglutination of cholera vibrios observed by them to be due to the causes producing Pfeiffer's reaction ...ed, in contrast to Gruber & Durham, referred merely in a tentative manner to the practical value of agglutination tests

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Further investigations on Pfeiffer's reaction and bacteriolysis

As shown by investigations of Baumgarten (1921), it is possible to use mice in place of guinea pigs when performing Pfeiffer s test However as noted by Hetsch (1928) the results obtained with the aid of this modification were not as uniform as those with Pfeiffer s original technique, presumably because complement, which is indispensable for bringing about the reaction is not as plentiful in mice as in guinea pigs

According to the summary of Hetsch (1928) the outstanding value of Pfeiffer s test carried out in the classical manner was confirmed by numerous observers. He noted in this connexion that while cholera immune sera failed to react with cholera like vibrios the latter reacted typically with sera manufactured with homologous or serologically identical organisms *Vice versa* the immune sera *raised in this manner gave negative tests with* cholera vibrios

Harvey (1929) though admitting that Pfeiffer s reaction had been largely superseded by agglutination tests upheld its value " in difficult sporadic cases of cholera like disease " In view of the availability of the highly specific O sera it is a moot point whether this statement is still valid at present.

In the course of his investigations Pfeiffer devoted attention to the question of to what extent his reaction could be produced outside the living organism

Pfeiffer (1894b) established in this connexion that, if a broth suspension of cholera vibrios mixed with a dose of potent immune serum was injected into the peritoneal cavity of freshly-killed guinea-pigs and the carcases were kept in the incubator marked bacteriolysis took place for the first 20 minutes but did not progress further

As Pfeiffer observed in 1895 dilutions of cholera-immune serum in broth, which proved highly vibriolytic when injected into the peritoneal cavity of guinea-pigs, exerted *in vitro* no bactericidal action on cholera vibrios and even formed a suitable substrate for their multiplication. If however a 1 / dilution of cholera immune serum in broth was injected intraperitoneally and droplets of the peritoneal contents of such animals removed after 20 minutes were seeded with cholera vibrios, bacteriolysis took place but often did not become complete.[1]

In vitro bacteriolytic tests, to be used side by side with or in place of Pfeiffer s reaction have been recommended by several authors.

Thus Serkowski (1906) mixed various saline dilutions of the sera to be tested with constant quantities of cholera vibrios and of complement (normal serum) and, after incubation at 37°C for 4-6 hours, used the mixed material for pouring agar plates which were kept in the incubator for 24 hours. The absence of growth, or the number of colonies which had by then developed, indicated to what extent the serum dilutions in question possessed bacteriolytic properties.

Using a similar technique Amako (1909) added to two parts of the various serum dilutions to be tested 1 part of a suspension of cholera vibrios and 1 part of normal rabbit

[1] This method was also used by Bordet (1895). Craster (1914), giving priority to the latter worker stated that he had utilized Bordet test to demonstrate bacteriolysis *in vitro* side by side with Pfeiffer reaction for study of cholera-like vibrios.

Though as summarized by Hetsch (1912, 1928) the question of the interrelations existing between the above-described antibodies as well as the problem of their immunological importance soon became the subject of considerable dissensions, early practical experiences, particularly the large-scale investigations of Kolle & Gotschlich (1903) endorsed the specificity and consequently the diagnostic importance of the reactions concerned, particularly of the agglutination test

A somewhat divergent opinion was expressed by Friedberger & Luerssen (1905) who claimed that the usefulness of the latter method was limited in that in the case of freshly isolated strains after an incubation at 37 C for 6-8 hours, the vibrios showed spontaneous agglutination ("pseudo-agglutination") in normal saline Since however in actual practice agglutination tests are made after longer intervals (at an average after about 18 hours) when, as admitted by Friedberger & Luerssen, spontaneous agglutination had become absent their observations would be of little importance even if generally valid However as shown by subsequent investigations especially by the experiences of Kabeshima (1918c) who examined 160 freshly isolated cholera strains in this respect with negative results, the phenomenon of pseudo-agglutination observed by Friedberger & Luerssen in the case of only 11 strains, must be rare

As already alluded to in Chapter 3 the discovery of the El Tor vibrios (Gotschlich 1905 1906) which, though giving serological reactions identical with those of the classical *V cholerae* appeared to be different from the latter on account of their apathogenicity and their haemolytic properties, led to most serious dissensions in regard to the specificity of the serological tests Ruffer (1907) one of the protagonists of the school claiming a separate status for the El Tor vibrio went so far as to conclude

"That it is not advisable to trust to the agglutination test only in the bacteriological diagnosis of cholera. The test is useful but not specific."

The German workers, on the other hand (see summary by Kolle & Schürmann, 1912) who denied the existence of qualitative differences between the cholera and El Tor vibrios, continued to maintain that

"the system of cholera diagnosis which is based largely upon the immunological reactions, still rests upon a fully secure scientific foundation and has as well proved its practical value" [Trans.]

Though it remains legitimate to evaluate the differences existing between the classical cholera vibrio and the *V El Tor* either in favour of the unity of the two or to support the concept of their separate standing modern investigations have left no room for doubt that, as far as their basic serological reactions are concerned, the two organisms do not differ Evidence in this respect, additional to that furnished in the preceding section of the present disquisition, will be brought forward in the following pages.

It may be conveniently added that, as recorded by Popescu (1924) the washed blood platelets of cholera immunized rabbits produced not only agglutination but also lysis of cholera vibrios It is also of interest that, according to the experiences of Pacheco & Peres (1940) mucin reduced or even inhibited the vibriolytic action of cholera immune sera (inactivated by heating at 55 56°C) in the presence of complement. The differences between the lytic action of cholera immune sera and that produced by bacteriophage were studied by MacNeal Frisbee & Krumwiede (1937) In contrast to the former bacteriophage lysis was transmissible in series and rapidly led to variations in the size and form of the vibrios

General agreement exists that bacteriolysins are present in the sera of normal persons at rather low titres only Papamarku (1917) noted in this connexion that while some previous observers met usually with titres varying from 0 1 to 0 75 in the sera of their controls, he found that 3 out of 16 such normal individuals had bactericidal titres of 0 05, while the others had titres below this figure.

As summarized by Svenson (1909) the presence of bacteriolysins in the sera of cholera patients or convalescents was demonstrated by several early workers such as Lazarus (1892) Metchnikoff (1893) Pfeiffer (1894a) and Amako (1909) who as a rule resorted to animal experiments, especially Pfeiffer s test, but occasionally relied only upon *in vitro* tests. As found by these and other observers the bactericidal titre of such sera (i e., the minimal amount still protecting guinea pigs against intraperitoneal cholera infection) varied considerably and was, according to Metchnikoff even in the case of past severe infection sometimes not higher than that of normal sera. In the experience of this worker during the acute phase of the disease bactericidal substances were present in but small amounts in the sera of not more than 45% of the sufferers However as established by him and all other observers the bacteriolytic titre rose during convalescence, to become maximal usually during the second and fourth week. In the experience of some but not of all workers quoted by Svenson, the protective value of the convalescent sera was not higher than that of normal sera after 6 weeks

Svenson himself was able to demonstrate the presence of bacteriolysins in 89% of the 27 convalescents examined by him within the second to fourth week after onset of the disease There was no parallelism between the presence of bacteriolysins and that of agglutinins found in the sera of only about one third of these persons In view of the fact that Pfeiffer s test was negative in the case of some convalescents who had survived severe cholera attacks, Svenson concluded that the appearance of bacteriolysins because frequent, was a characteristic sign of recovery but did not fully account for it (*eine Begleiterscheinung die sehr häufig bei der Genesung beobachtet wird und ein charakteristisches Symptom derselben ist mit derselben aber nicht unbedingt identifiziert werden darf*)

serum diluted 1/10 The tubes were kept at 37°C for 1 hour then smears were made and stained with dilute carbol fuchsin in order to determine to what degree bacteriolysis had taken place. At the same time amounts of 0 01 ml from each tube were used to pour gelatin-agar plates. Colonial counts were made after an incubation at 37°C for 24 hours.

Prausnitz & Hille (1924) besides confirming the above-mentioned observations of Pfeiffer also found it possible to reproduce the phenomenon of bacteriolysis *in vitro* with the aid of adequately-graduated amounts of immune serum and complement, particularly if fresh complement was added from time to time. Bacteriolysis became still more marked if also limited amounts of an exudate, which had been obtained through intraperitoneal injection of a guinea-pig with sterile broth, were added to the tubes.

An *in vitro* test for bacteriolysis with the aid of peptone water has been described by Kiribayashi (1931b) As summarized in the *Tropical Diseases Bulletin* (1932), his technique was as follows

" A loopful of a 20-hour agar culture of test vibrio is suspended in peptone water (peptone 3 sodium chloride 5 dist. water 1 000) of pH 7 6 which is isotonic with the serum components of the test. A comparison is made by setting up two sets of dilution mixtures, the one containing inactivated immune serum, complement and suspension of test organism and the other a control, containing inactivated rabbit serum, complement and suspension. Specific bacteriolysis is indicated after 3-5 hours by the absence of turbidity in the first set of mixture and positive turbidity in the control set."

Gordon & Johnstone (1942) stated that with the aid of bactericidal tests with normal guinea-pig serum it had been possible to detect antigenic differences between true cholera strains and cholera like vibrios and even to single out cholera strains which differed antigenetically from the main group (see also preliminary observations made in the latter respect by Mackie & Finkelstein, 1931) In the opinion of Gordon & Johnstone, the bactericidal technique " may be of use in differentiating between strains of *V cholerae* for which the agglutination technique is not practicable, and as an alternative method to the haemolytic test for distinguishing between strains of *V El Tor* and true cholera strains."

Ahuja (1951) and Singh & Ahuja (1951) noting that fresh guinea pig serum exerted a marked vibriocidal effect on rough or partially rough cholera vibrios, while leaving smooth vibrios unaffected, recommended the following test for the detection of roughness in *V cholerae* strains

An 18-hour-old peptone water culture of the strain to be tested was diluted 100-fold with the same medium. One part of this dilution was mixed with two parts of 1 in 2 complement (diluent peptone water). The mixture was incubated for 4 hours at 37°C. The initial inoculum and the 4 hours growth in the presence of complement were then sampled by plating 3-mm loops on agar plates without spreading, and results were read after incubation at 37°C for 18 hours.

It was found that rough or partly rough cholera vibrios either failed to grow or grew to a greatly reduced degree in the presence of complement, an effect which was inhibited by heating the mixtures at 56 C. Since 20% of the guinea pig sera tested did not exert this effect, it was essential to make preliminary tests with known smooth strains.

While Gallut (1953a) did not consider the above-described test fully reliable, Dudani (1955), after an examination of 51 cholera growths, reasserted the value of the method of Singh & Ahuja.

(or agglutinins) in the sera of healthy carriers even within the days immedi ately following the isolation of *V cholerae* from their faeces reached on the contrary the conclusion that the freedom of the carriers from clinical manifestations of the infection could not be the result of a general immunity but must have depended upon other factors possibly a local immunity

Levi della Vida (1913) though able to demonstrate the presence of agglutinins in all but 9 of the 48 convalescent and healthy carriers examined by him found bacteriolysins less regularly demonstrable so that they were absent from some of the well agglutinating samples On the other hand bacteriolysins were never detected in sera devoid of agglutinating power

Sano (1921) found like de Bonis that the sera of healthy cholera carriers contained practically no immune bodies, while Toguchi (1919) as quoted by Takano Ohtsubo & Inouye (1926) recorded that " the immunological reactions of the carriers are not uniform and in some cases they are not stronger than those of the healthy person " In accord with de Bonis Toguchi assumed that the absence of clinical manifestations of cholera in the carriers must " be explained on some basis other than the immunological reactions of the blood serum " A similar opinion was expressed by Satake (1926) who was able to demonstrate the presence of bacteriolysins in but one out of 5 cholera carriers and apparently found agglutinins in none of them

Classical investigations by Kolle (1896 1897) showed that administra tion of killed cholera vaccines in a single high dose (1/10th of a culture) to 17 individuals led to a most marked increase of the originally almost invariably low bacteriolytic titre of their sera. As far as could be established, the increase of the titres became manifest already 6-10 days after vaccina tion and was still demonstrable for periods up to about 12 months (350 days) after the vaccine had been administered

While several of the subsequent observers maintained in agreement with Kolle that a rise of the bactericidal titres did not become demonstrable before the fifth day after vaccination, some noted an earlier increase of the bacteriolysins Balteano & Lupu (1914) examining two individuals vac cinated for the first time with doses of 1 ml spoke in this connexion of the fourth day after administration of the prophylactic. Aaser (1910) drew attention to three persons in whom a considerable rise of the bacteriolytic titres (twice to 0 01 once even to 0 003) had been noted already on the third day after vaccination Ahuja & Singh (1948) examining 21 persons once inoculated with 1 ml of cholera vaccine likewise established that the sera of these individuals showed vibriocidal properties after three days

It is unanimously stated that the initial appearance of bacteriolysins in the sera of cholera vaccinated persons is followed by a further increase of the bacteriolytic titres However the statements as to when their maxima

Referring to observations made in regard to the persistence of bacteriolysins in the sera of persons who had recovered from cholera Papamarku (1917) stressed that obviously these immune bodies were apt to become inconspicuous or even absent at a time when the individuals in question were still fully immune against the disease A case in point was that of Pfeiffer whose serum showed no specific immunizing properties only three months after he had been affected by cholera

Further noteworthy observations on the presence of bacteriolysins in the sera of cholera patients or convalescents may thus be summarized

Shiiba & Oyama (1920) demonstrated the presence of bacteriolysins in the sera of 97 convalescents with the aid of the Neisser and Wechsberg test. As a rule, though not invariably the results thus obtained ran parallel with those of agglutination tests.

Tagami & Watanabe (1920) also applying Neisser and Wechsberg s method, obtained positive results in 89 / of the 91 sera tested. Bacteriolytic tests were found to yield results earlier than agglutination tests, becoming positive in 80 / to 85 / of the cases in 1 to 3 weeks. The bacteriolytic titres decreased after the third week, only half of the sera still proving positive after one month. In the experience of these two workers, " the agglutination and bactericidal reactions do not run parallel, but sometimes quite oppositely "

Ukil (1928) studying 30 convalescent sera collected from cholera patients in Calcutta, 25 of which agglutinated the causative organisms at titres ranging from 1 100 to 1 1000, found that 18 of these sera possessed marked bacteriolytic properties, while seven produced less marked, and five weak reactions in tests made *in vitro*

Continuing such tests (platings from tubes containing a mixture of two drops of the convalescent serum under test, 4 drops of a suspension of *V cholerae* containing 2000 million organisms per ml, 2 drops of 50 / complement and 0 6 ml normal saline, which had been incubated at 37°C for 4 hours) Ukil & Guha Thakurta (1930) found that the bacteriolytic properties of the convalescent sera increased progressively reaching their maximum at the time when the faeces of the individuals tested no longer yielded positive cultures, i.e., usually 1 3 weeks after onset of the disease.

As far as it was possible to continue the observations, it appeared that the bacteriolytic properties of the convalescent sera remained manifest for several weeks. The presence of bacteriolysins was confirmed by rabbit experiments, a dose of 0.5-1 ml intravenously as a rule protecting the animals against intravenous injection of a lethal dose of *V cholerae* It was found that 85 / of the convalescent sera tested agglutinated cholera vibrios at titres ranging from 1 100 to 1.3200

Attempts to demonstrate the presence of specific bacteriolysins in the sera of cholera patients with the aid of a modified Pfeiffer test gave according to a statement made in the 1941 report of the Indian Research Fund Association, no fully satisfactory results. Though sometimes present early in the disease, bacteriolysins could be demonstrated in but 33 / of the sera examined. In a majority the bacteriolysins showed no type specificity

Observations on the presence of bacteriolysins in the sera of healthy cholera carriers are not numerous. Massaglia (1911) claimed that bacteriolysins as well as agglutinins were present in the sera of such individuals in the same amounts as in the sera of convalescents, and postulated therefore, that the freedom of the carriers from manifest signs of cholera was due to the presence of immune bodies in their blood De Bonis (1912) since he was unable to demonstrate bacteriolysins

20 days after administration of an equally high initial dose a rapidly setting in but temporary drop of the bactericidal power It is of great interest that Papamarku (1917) revaccinating guinea pigs three weeks after an initial administration of cholera vaccine also noted such a drop of bactericidal power but established that nevertheless most of the animals in question resisted during the persistence of this " negative phase " intra peritoneal challenge with lethal doses of *V cholerae*

Haemolysins

Supplementing the information already furnished in the third chapter it has first to be noted that though giving consistently negative results in standard haemolytic tests, according to some workers the classical cholera vibrios were able to lyse goat and sheep red blood corpuscles under peculiar conditions The following observations have to be recorded in this connexion

Doorenbos (1932) claimed that it was possible to transform through bacteriophage action non-haemolytic cholera vibrios into haemolytic vibrios which thus showed the properties of *V El Tor* Having also found that 24-hour-old cultures of non haemolytic cholera vibrios possessed anti-haemolytic properties, being capable of inhibiting haemolysis of sheep erythrocytes through El Tor vibrios, Doorenbos reached the conclusion that " the presence or absence of haemolytic properties depends solely upon the proportion of haemolytic and anti-haemolytic elements present in the strain in question"

Reporting in a later article upon the examination of 12 cholera strains which had been isolated one to two months previously at Calcutta from the dead bodies of cholera victims and had been forwarded to Alexandria, Doorenbos (1936a) stated that all these strains were incapable of producing haemolysis of sheep erythrocytes after cultivation for 24 hours, but that after incubation for only 8 hours, 4 of the strains exerted a marked and 5 a feeble haemolytic action.[1]

Doorenbos (1936b) further maintained that, in analogy with the findings made in the case of *V cholerae* young (6-8 hours old) El Tor cultures showed more marked haemolytic properties than those grown for 24 hours. He also claimed the existence of strains which, on account of their feeble haemolytic properties, stood half-way between the classical *V cholerae* and the true El Tor vibrios.

Vassiliadis (1935b) stated that cholera vibrios which had been cultivated in broth containing 5 per 1000 glucose, showed in contrast to those grown in ordinary broth haemolytic properties for sheep erythrocytes and also recorded (1935a) that two originally non-haemolytic cholera strains produced marked haemolysis after they had been passed three times through ordinary broth and subcultivated for a fourth time in glucose broth. Vassiliadis further stated (1935b) that he had been able to produce through immunization of rabbits not only with El Tor vibrios but also when using instead a non-haemolytic cholera strain for this purpose, anti-haemolysins inhibiting the haemolytic properties of filtrates from El Tor cultures. He also claimed that the cholera immune serum routinely used in his laboratory for agglutination tests neutralized the El Tor haemolysins at the same titre as anti-haemolytic El Tor sera. Vassiliadis concluded from these observations that a non-active haemolytic antigen was present in the classical *V cholerae*

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[1] The occurrence of a haemolytic phase becoming manifest in otherwise typical cholera strains during the sixth and twelfth hours of growth on agar has been recorded again by Fournier (1940).

are reached and as to how long the titres remain at this level vary considerably Thus according to Ahuja & Singh (1948) the maximum of the bacteriolytic titres was attained about the 8th day after vaccination and a fall of the titres became manifest by the 30th day Sano (1921) noted that the bactericidal titre in the sera of cholera vaccinated persons reached an acme in three weeks, while Balteano & Lupu (1914) found that in two persons vaccinated against cholera for the first time the bacteriolytic titre became maximal not sooner than after 56 days

Though it is generally agreed that the drop of the bactericidal titres setting in after their maximum had been reached and persisted for some time is gradual, observations regarding the length of the period during which bacteriolysins remain demonstrable in the sera of cholera vaccinated individuals at increased titres gave divergent results As noted already Kolle found in some of the persons vaccinated by him conspicuous bactericidal titres even after a period of about 12 months Sano (1921) noted that these titres fell as low as they were in normal blood sera within 10 months Hetsch (1928) summarizing the observations made in this respect during the First World War maintained that as a rule the increase of the bacteriolytic titres persisted not longer than 7-8 months In the experience of Ahuja & Singh (1948) the level of the bacteriolysins in the sera of cholera vaccinated individuals was by the 100th day already but slightly higher than before immunization Papamarku (1917) examining 60 persons at varying times after cholera vaccination, noted that Pfeiffer tests, made with the sera collected from 31 of these individuals between the 11th and 134th day after immunization were positive in 61%, whereas an identical result could be obtained in only 8% of 28 persons tested during a period of from 146 days to about 300 days after vaccination.

The few observations made in regard to changes in the bactericidal titres as a result of revaccinations against cholera gave strikingly divergent results. Balteano & Lupu (1914) while maintaining that in the two persons who had received only a single dose of cholera vaccine a temporary drop of the immune bodies, including the bacteriolysins, was noticeable stated that in those persons who had received one or two further doses at weekly intervals no such " negative phase " was present, the bacteriolytic titres rising rapidly and reaching a maximum of 1 150 which was maintained up to the end of the observation period of three months Karwatzki (1906b) found that the bactericidal power of the sera of 11 individuals, which did not greatly increase after the first administration of cholera vaccine, was most markedly enhanced after a second dose had been given five days later Ahuja & Singh (1948) stated on the contrary that injection of a booster dose six months after the initial cholera vaccination did " not increase the vibriocidal power of the serum to any marked extent compared to the effect produced by the primary stimulus " Aaser (1910) even noted in the case of one person who had been revaccinated with 2 ml of cholera vaccine

Bernard Guillerm & Gallut reached the tentative conclusion that cholera and El Tor vibrios possessed a common haemolysin which was free in the latter organisms combined with a neutralizing substance in *V cholerae* They noted that with the aid of acetone one could extract from both organ isms a haemolytic substance which was soluble in ether and warm alcohol insoluble in benzene and thermostable when heated for 10 minutes at 100 C Emulsified in saline at a pH of 7 2 to 8 0 it haemolysed living vibrios This substance which consisted of water insoluble fatty acids appeared to play no role in the usual phenomena of haemolysis

In analogy with the above findings, Read, Pandit & Das (1942) through an exhaustive investigation of 62 strains of classical cholera, El Tor and cholera-like strains already referred to in Chapter 3 reached the following conclusions

"(a) The strains can be divided into two groups the early haemolytic and the late haemolytic organisms, corresponding to Greig positive and Greig-negative organisms. In the former group haemolysis is usually complete within a few minutes to 24 hours, is not markedly affected by performing the test at 12 C or under reduced oxygen tension and production of the haemolysin is not affected by exclusion of oxygen. In the latter group haemolysis is usually partial, hardly occurs in 24 hours and is abolished when the test is performed at 12 C or under restricted oxygen supply

"(b) Antihaemolytic sera prepared from Greig positive organisms have a definite specific neutralizing effect on the haemolysin of the early haemolytic group No similar effect has been demonstrated in the late haemolytic group."

In the opinion of Read and his colleagues the "early" haemolysins were most likely identical with van Loghem s exohaemolysin, the "late" haemolysins with the haemodigestive ferment described by this worker

Investigating recently three El Tor strains, one identical strain from Celebes, and 10 strains of water vibrios, all but two of which fell into Heiberg s carbohydrate group I Brück & Brandis (1953) found that all these growths produced a soluble haemolysin demonstrable in Berkefeld filtrates but not in Seitz filtrates of 3-day-old broth cultures Though thermolabile upon prolonged heating at 50 C application of this tempera ture for a time sufficiently long to kill the vibrios (e g for 15 minutes) did not inhibit the action of this haemolysin for sheep erythrocytes Likewise, the haemolysin was not inhibited by ultrasonic vibration sufficiently in tense to kill the vibrios

As already referred to in the preceding chapter Zimmermann (1934) was able to establish that most of the classical cholera strains examined by him were capable of producing thermolabile haemolysins for human erythrocytes whereas the El Tor haemolysins were active not only against these but also against sheep red blood corpuscles As noted, these findings have been recently confirmed by De and co-authors (1954) through an examination of 27 cholera, 2 El Tor and 14 cholera-like strains It was found in the course of this investigation that calcium while inhibiting the

It is noteworthy however that in the experience of Goyle (1939) immune sera raised against typical non-haemolytic *V cholerae* exerted no neutralizing action on the haemolysins of El Tor or other haemolytic vibrio strains.

Van Loghem (1925) studying 14 cholera and 4 El Tor strains, reached the conclusion that a haemolysin was produced by the latter organisms which reacted like an *exotoxin* it was soon demonstrable in culture filtrates, was thermolabile and injected into rabbits, produced an anti haemolysin as previously shown by Kraus & Přibram (1906) The haemolysin of *V cholerae* appeared much later in the cultures and had the character of an *endotoxin* being neither thermolabile nor antigenic It was possible to demonstrate the presence of such an endotoxin also in old El Tor cultures.

Van Loghem recorded in a further communication (1926) that bacteriophage action hastened the liberation of the cholera endohaemolysin whereas in filtrates of cultures, which had been acted upon by bacteriophage, marked haemolysis was demonstrable in 5-6 days in the filtrates of cultures subject to autolysis only this phenomenon became apparent not sooner than after 8 or 9 days

The observations of van Loghem were confirmed and amplified through interesting studies of Bernard, Guillerm & Gallut (1939a 1939b 1939c)

Bernard and colleagues (1939a) were able to extract with the aid of ammonium sulfate from 3-day-old El Tor agar cultures and even from 24-hour-old broth cultures or saline suspensions a substance which, redissolved in normal saline proved strongly haemolytic for sheep erythrocytes. It was not possible to extract such a haemolysin from cholera cultures but a feebly haemolytic substance could be extracted from saline suspensions or broth cultures of *V cholerae* which had shown evidence of haemolysis after incubation for 5 and 9 days respectively

In their second note (1939b) Bernard and co-workers recorded that addition of suitable quantities of El Tor or cholera vibrios to a 2.5 % suspension of sheep blood corpuscles led to the production of a violet colour Due apparently to a reduction, this phenomenon could not be produced by vibrios which had been killed by heat (56°C) or by alcohol.

Bernard and his colleagues maintained in this connexion that haemolysis of *V El Tor* was facilitated by an optimum relation between the number of organisms and that of erythrocytes, the violet decoloration remaining absent in this case Both an excess and too small an amount of blood corpuscles retarded haemolysis. The violet decoloration was marked in the former case, but disappeared as soon as lysis took place.

In a third communication (1939c) these authors stated that

(1) the exohaemolysin of *V El Tor* was inactivated by heating for 1 hour at 56°C or for 5 minutes at 100°C, was destroyed by ether or formol and neutralized by cholesterol, but not affected by toluene and activated by lecithin (egg yolk)

(2) the endohaemolysin of *V cholerae* acted slowly producing the brown colour of methaemoglobin, was destroyed by 5 minutes heating at 100°C, neither inhibited by cholesterol nor activated by lecithin

(3) mixtures made in varying proportions of (*a*) the endohaemolytic substance of *V cholerae* inactivated by heating, and (*b*) the active exohaemolysin of the El Tor vibrio were apt to inhibit the haemolytic action of the latter organism. Haemolysis was apt to take place, if an excess of El Tor haemolysin was used, but led to the production of methaemoglobin

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haemolytic activity of El Tor and cholera like vibrios, was essential for that of *V cholerae*

When trying to evaluate the above-discussed observations in conjunction with those described in the third chapter, one may claim that, as far as their behaviour in blood-containing media is concerned not merely quantitative, but distinctly qualitative differences exist between the classical cholera and the El Tor vibrios. Since, however the *V cholerae* is to some extent endowed with haemolytic properties it appears to be at the same time more a question of personal bias than of factual evidence whether as far as their behaviour in blood-containing media is concerned it is justified to place the two organisms into two different species instead of considering them as variants of one species

Agglutination

(A) Identification of suspect strains

As can be gathered from the publication of Gruber & Durham (1896) these pioneer workers used for the identification of suspect strains with the aid of agglutination tests sera prepared by intraperitoneal immunization of guinea pigs with killed cholera cultures Dilutions of these sera in broth and broth suspensions of the organisms to be tested were used for both microscopic and macroscopic examinations. To carry out the former, drops of the diluted sera and of the suspensions were mixed on cover glasses and the latter were mounted on hollow slides. Macroscopic observations (checked if necessary under magnification) were made by mixing 0.5-ml quantities of the diluted test sera and of the vibrio suspensions. Moreover a microscopic preliminary test (*Vorprobe*) was recommended for which, instead of drops of suspensions made from pure agar cultures, loopfuls taken from the top-most layer of the primary stool cultures in fluid media were used after the presence of motile organisms had been ascertained. As stated by Gruber & Durham it was possible under favourable conditions to obtain in this manner a fairly conclusive result within 6 to 10 hours.

Systematic studies in the Berlin Institute for Infectious Diseases led to a considerable refinement and a standardization of the agglutination technique As summarized by Kolle & Gotschlich (1903) it was found necessary to use for the manufacture of immune sera rabbits or donkeys in preference to horses or goats, the normal serum of which agglutinated cholera vibrios at a higher titre (1 40-1 50) than the rabbit and donkey sera, the titres of which were 1 10 and 1 20 respectively It was also indispensable to use instead of broth apt to give inconstant results in successive tests, normal saline for the dilution of the sera and for suspension of the test organisms Though, as stated in an instruction for the laboratory diagnosis of cholera by Koch et al originally issued in 1902 and revised

in 1904 and 1907 (see text reprinted by Kolle & Schürmann, 1912) it was permissible to use hanging-drop preparations (inspected at low magnification and not under oil immersion) as well as macroscopic tube tests for this work results obtained with the former method could be considered final only if quite clear-cut (*über allem Zweifel eindeutig*—Kolle & Gotschlich 1903)

A modification of the agglutination technique introduced soon after these studies was the use of rapid slide tests for the preliminary identification of cholera suspect colonies or growths As Costa (1912) claimed without furnishing a reference, Salimbeni was the first to take advantage of this expedient and now amply used procedure in cholera diagnostic work To judge from a remark made by Sierakowski (1920a) an early recommendation of this method was also made by Bujwid

Like Gruber & Durham Dunbar (1905) suggested a rapid method for the serological identification of *V cholerae* according to which mucous particles of suspect stools, emulsified in drops of peptone water on cover slips, were mixed with drops of diluted (1/500) high-titre cholera serum or for the purpose of control with normal rabbit serum, diluted 1/50 As claimed by Dunbar in positive cases the motility of the vibrios was soon inhibited by the specific serum or it was even possible to observe agglutination However as stated by Kolle & Schürmann, tests made in the Berlin Institute for Infectious Diseases failed to confirm the value of this method. It was possible to obtain occasional positive results through agglutination tests with drops which had been removed from the peptone water cultures used for preliminary enrichment after 5 hours incubation i.e. by a method analogous to the *Vorprobe* of Gruber & Durham Similar procedures were also recommended by several subsequent workers without reference to the original method of these two observers

An alternate method recommended by Bandi (1910) for the rapid laboratory diagnosis of cholera was preliminarily to add adequate amounts of specific serum to 5 ml peptone water samples filled into special drawn out test tubes. If these were then inoculated with cholera-suspect stools and incubated at 37 C according to Bandi s observations in positive cases clumps of agglutinated vibrios could be seen in the lowest part of the tubes after 2 7 hours [1]

The usefulness of Bandi s method or of similar procedures for the rapid serological diagnosis of cholera has been endorsed by several observers Thus as late as 1951 Cossery made the following statement

"I have used Bandi s test routinely for the examination of cholera-suspect vibrios since 1918

[1] It is of historical interest that Achard & Bensaude (1897), besides making direct agglutination tests, used method similar to that later recommended by Bandi to demonstrate the presence of agglutinins in the sera of cholera patients by cultivating cholera vibrios in 10 drops of broth to which 1 drop of serum of the patient in question had been added As noted later an analogous method was also applied by Ransom & Kitashima in 1898

"During this long time I have used it in many thousands of cases. It has always given me a fairly satisfactory result if the two following conditions are observed

"(1) A high-titre serum is used, of which the final dilution in the peptone solution is about 1 100

"(2) The tubes are not shaken while in the incubator or while getting them out for reading."

Parallel tests made by Ghosal & Paul (1951 1952) with the aid of Bandi s method and with two highly specific media now available—which, as will be described in a later chapter give fully satisfactory results if used for direct platings from cholera-suspect stools—failed to confirm the out standing value of the former test. As established under experimental conditions, Bandi s test was useful in the diagnosis of cholera when *V cholerae* preponderated in the stools whereas a preponderance of coliform organisms exerted an adverse effect upon the results In the actual examina tion of 285 stool samples the cultural method was found to give 38% more positive results than Bandi s method

Considering this evidence the WHO Expert Committee on Cholera (1952)

"came to the conclusion that Bandi s test did not give sufficiently reliable results to recommend its adoption for the laboratory diagnosis of cholera" [Page 4]

Studying 81 cholera and 31 El Tor strains, Gispen (1937 1939) found that the O-agglutinability of alkaline saline suspensions of the former organisms which had been heated for three hours at 56 C, b came as a rule inhibited or at least markedly reduced, whereas El Tor susp nsions did not exhibit such a thermolability The agglutinability of the cholera vibrios could be restored by prolonged heating or by the addition of broth or peptone to the heated suspensions In Gispen s opinion the differences in agglutinability observed by him could be ascribed to the presence of different proteins in the cholera and El Tor vibrios respectively according to the observations made by Linton (1935) [1]

De Moor (1939) expressed doubts regarding the diagnostic value of Gispen s reaction, stating that it

"may show up striking differences in a great number of cholera and El Tor strains, but the estimation of it in a given case is more difficult than Gispen pretends. Gispen mentions cholera strains that present the phenomenon either not or less distinctly It appeared here that cholera strains which one time had become in- or hardly agglutinable, would another time lose hardly any of their agglutinability in the O-cholera antisera Inaba or Ogawa."

As recorded in 1948 by de Moor s assistant, Tanamal, a difference between cholera and El Tor vibrios could be demonstrated by using a potent cholera O serum free from preservatives and diluted 1/200 to

[1] As stated in footnote to an article by de Moor (1949), F H. Meyer claimed in a thesis published at Amsterdam in 1939 to have observed differences in agglutinability identical with those recorded by Gispen, when treating suspensions of cholera and El Tor vibrios with 2% chloroform.

which 0.3% of sodium carbonate had been added Cholera vibrios if added to such a serum in a dense suspension in distilled water, did not become agglutinated, while the agglutinability of El Tor vibrios was not impaired by addition of the chemical

It is of interest to add that, as also noted by Tanamal, cholera vibrios added in a dense suspension to a 0.5% solution of sodium bicarbonate to which after 15 minutes an equal volume of 0.5% mercuric chloride was added, became precipitated, whereas El Tor vibrios remained in suspension

Sharing the opinion of Gispen de Moor (1949) ascribed the above mentioned differences between cholera and El Tor vibrios to the different nature of their protein fractions

(B) Tests with human sera

As noted already Achard & Bensaude reported soon after Gruber & Durham had recommended agglutination tests for the identification of unknown cholera strains, that this method could be used as well to demonstrate with the aid of known cholera cultures the presence of agglutinins in the sera of cholera patients

The initial observations of these two workers have been followed up and amplified by numerous investigators who have devoted attention to the presence of agglutinins not only in the sera of cholera patients and convalescents but also in normal persons including those who had been vaccinated against cholera and in carriers The results of these studies may thus be summarized

(1) *Normal (cholera-free) individuals* As summarized by Karwatzki (1906b) and Greig (1915) in the experience of the earlier workers the normal sera of cholera free and non vaccinated individuals almost invariably agglutinated the *V cholerae* if at all, at titres not exceeding 1 10 or at most 1 20 Exceptions to this rule have remained extremely rare Krishnan & Dutta (1950) being apparently the only observers definitely stating that they had observed once a higher titre (1 80) in a "normal" group of 18 persons tested before they received cholera vaccination. Three other members of this group showed agglutinating titres of 1 10 only while the sera of the 14 failed to agglutinate either Inaba or Ogawa suspensions

(2) *Cholera patients and convalescents* The following of the fairly numerous observations on the presence of agglutinins in the sera of cholera patients or convalescents deserve attention for the purposes of the present disquisition

Achard & Bensaude, reporting in 1897 on a total of 14 observations, stated that they had found agglutinins in 13 of these instances—12 times during the stage of attack (first to sixth day) when the agglutination titres ranged from 1 25 to 1 50, and once in a convalescent examined for the first time on the 28th day after the onset of cholera and then showing a titre of 1 120

The first studies on somewhat larger groups of cholera patients or convalescents seem to have been published in 1909 by Amako Kopp and Svenson.

Amako (1909) tested the sera of 58 cholera-affected individuals with the aid of tube tests, adding 2 mg respectively of a cholera culture to 3 ml of the various serum dilutions, and reading the results after an incubation at 37°C for 3 hours. While results during the first week after onset were negative, the agglutination titres became maximal during the second week and then decreased. The titres ranged in slight cases from 1 40 to 1 80 in moderately severe cases from 1 20 to 1 640, in severe cases from 1 160 to 1 640 No agglutination was observed in cases ending fatally or in comatous patients.

Kopp (1909), studying 32 patients admitted to a hospital in St. Petersburg, Russia, obtained positive results with agglutination tests in 26 the titres usually ranging from 1 10 to 1 50, but rarely reaching 1 100 and usually becoming maximal in the second or third week after onset. No relation seemed to exist between the character of the attacks and the height reached by the agglutination titres.

Svenson (1909) testing 37 sera of cholera patients or convalescents with a technique similar to that of Amako, but taking readings already after incubation for one hour and again after the tubes had been kept at room temperature overnight, obtained only 13 positive results. The agglutination titres, which seemed not to be influenced by the character of the cholera attacks, remained invariably low reaching or approaching 1.50 in 8 instances, 1.25 in 5 instances. Positive results were obtained with specimens taken between the fifth and 60th day after onset of the disease, but only in rare instances before the 10th day Evaluating his own and Kopp s results, Svenson emphasized that the agglutinatory power of the sera from cholera patients or convalescents " is apt to be low and frequently not different from that of normal human sera "

In contrast to this postulation of Svenson, some other of the earlier workers observed occasionally that the sera of cholera convalescents agglutinated *V cholerae* at higher titres, the maxima recorded by Liveriato (1914) and Kabelík (1915) being 1 5000

Salimbeni (1915) examining 27 cholera patients or convalescents, laid stress upon the fact that the sera of six of these individuals who could be tested during or immediately after the attack, had neither agglutinating nor protective properties He concluded, therefore that recovery from cholera took place before antibodies had appeared in the blood-stream However though this postulation deserves attention, it has to be kept in mind that some of the workers mentioned above as well as some quoted below noted in part an increased agglutinin response during or soon after the acute stage of the disease

Commencing important observations on the presence of specific agglutinins in the sera of cholera patients or convalescents, Greig (1913a) found that two convalescents who continued to harbour cholera vibrios in their stools for considerable periods also produced positive agglutination reactions with their sera, whereas results of the latter test were negative in convalescents whose stools had become free from *V cholerae*

In 1915 Greig published the results of an examination of 363 sera derived mainly from cholera patients and convalescents, and to a lesser extent also from individuals in the stools of whom no *V cholerae* had been found. To carry out agglutination tests with these sera, mixtures were made in capillary tubes of equal amounts of the serum dilutions to be

tested and of suspensions of cholera vibrios in 0 85% saline, the results being read after an incubation at 37°C for two hours It was established through comparative tests that results of the agglutination tests were identical regardless of whether standard cholera strains or the homologous vibrios isolated from the convalescents in question were used

The outcome of Greig s large-scale study may thus be summarized

Nature of sera tested	*Number of sera*	*Result of agglutination tests*
From fatal cholera cases	64	Agglutinins absent in about half of these individuals even though some of them survived for 4 to 12 days. In the 23 positive cases agglutinins appeared comparatively late and almost invariably the titres did not exceed 1 40.
From recovering cholera patients	210	Agglutinins appeared rapidly in some instances as early as the second or third day of illness, and became as a rule well marked by the sixth day from onset. The titres, which varied from 1 400 to 1 1000, remained high up to the 17th day As far as could be gathered from scanty observa tions, a drop became marked about the 20th day
Patients from whose stools both cholera and cholera like vibrios had been isolated	18	Agglutination was positive only with *V choleræ* but not with the cholera-like vibrios in question.
Individuals in whose stools only cholera-like vibrios had been found	35	Agglutination tests with the homologous cholera-like strains were negative but in some instances positive results were obtained with *V choleræ* the individuals in question having obviously suffered from an unrecognized cholera attack.
Individuals in whose stools no vibrios had been found	36	Apparently for the reason stated above, some positive results were obtained in agglutination tests with *V choleræ* the titres at which reactions took place in this and the preceding group never exceeding, and but exceptionally reaching, 1 100.

Though realizing that agglutination tests were of little, if any, value for the diagnosis of acute cholera attacks, Greig stressed on the basis of the above recorded observations the importance of this method for a retrospective diagnosis of the disease

Shiiba & Oyama (1920) making agglutination tests with the sera of 97 convalescents found that as a rule a higher titre was reached after 2 3 weeks in the Japanese convalescents (1 80 to 1 640) than in the Chinese tested, in whom the titres ranged from 1 40 to 1 160 Only 7 out of the 97 persons composing this series had titres above 1 1000 with a maximum of 1 5120 in one instance

Tagami & Watanabe (1920) testing a series of 91 cholera convalescents, found agglutination to become positive in 87%, usually between the third and tenth day after the onset of illness rarely later up to the 15th day The agglutination titres ranged usually from 1 100 to 1 400 but exceptionally high titres, up to 1 10000 were said to have been met with occasionally Individually the highest titres were usually reached in 1 2 weeks, then a decrease set in, which was gradual at first but became more rapid after a week so that as a rule agglutination became negative one month after onset of the disease.

As referred to before, Ukil (1928) found that 25 out of the 30 cholera convalescent sera examined by him reacted positively in agglutination tests, the maximal titres being 1 100 in six instances, 1 500 in nine, and 1 1000 in ten. Supplementing this information in 1930 Ukil & Guha Thakurta stated that 15% of their convalescent sera failed to agglutinate *V cholerae* while 20% reacted at titres below 1 800 45% at titres ranging from 1 800 to 1 1600, and 20% at a titre of 1 3200

The studies on the agglutination reactions observable in cholera patients and convalescents by Pasricha, Chatterjee & Paul (1939) are of particular value because in contrast to the previous investigators, these workers could base their observations on the recent findings made in regard to the antigenic structure of *V cholerae* and the importance of the O antigens in immunological reactions.

Pasricha and colleagues used for their agglutination tests, made in Dryer s tubes suspensions of young cholera cultures to which 0 2% formol had been added as H antigens and boiled saline suspensions of *V cholerae* as O antigens Final readings were taken after the tubes had been kept for 18 hours at 55° C in a water bath.

Results of H and O agglutination tests made in this manner with the sera of 175 cholera patients (bacteriologically confirmed and non fatal cases) were recorded by Pasricha and his colleagues as follows

Day of illness	*Number examined*	*Number with chol agglutinins*	*Percentage showing H agglutinins*	*Percentage showing O agglutinins*
1st	19	0	0	0
2nd	13	0	0	0
3rd	17	0	0	0
4th	8	4	50	12
5th	8	4	50	37
6th	10	5	50	50
7th	13	8	61	46
8th	20	18	90	55
9th	24	18	71	60
10th	23	17	74	57
11th	9	8	90	66
12th	11	9	81	73
Total	175	91	52	

It will be gathered from this table that

(1) No agglutinins could be demonstrated in the sera of 49 patients of this series examined during the first three days of illness

(2) Becoming first manifest in the patients seen on the fourth day of illness, agglutinins were present in an on the whole increasing number of the sera from the seventh day of illness onwards

(3) O agglutinins appeared more tardily and were throughout the observation period present in the sera in a lesser percentage than the H agglutinins

It is in accord with this last observation that as shown elsewhere in the article by Pasricha, Chatterjee & Paul, the maximal titre at which O-agglutination took place was 1 320 as against 1 640 in the case of H-agglutination and that the average titres of agglutination were considerably lower than those of H agglutination

Further interesting points elicited by these workers were that

(a) In contrast to the postulation of Greig, agglutinins were found to be better developed for the homologous strains (isolated from the faeces of the patients in question) than for the standard Inaba strain of *V cholerae* used, agglutinins for the latter being absent in 11 of the 58 sera examined in this respect.

(b) However in 9 bacteriologically proven cases agglutinins, almost exclusively of the H type, were found for the standard strain, and no agglutinins for the homologous strains—a phenomenon for which "no satisfactory explanation" could be advanced.

(c) In conformity with Greig's findings, in patients showing both cholera and cholera like vibrios in their stools, agglutinins were demonstrable only for *V cholerae* Such agglutinins were also found in a few patients harbouring only cholera-like vibrios in their stools as well as in some patients with clinical signs of cholera but negative bacteriological findings.

In regard to the last mentioned point, it is, however important to add that, according to findings recorded in the 1941 report of the Indian Research Fund Association in some instances where both cholera and cholera like vibrios had been isolated from the stools, agglutinins for both kinds of organisms could be demonstrated in the sera of the patients in question

Referring to further observations on the appearance of O antibodies in the sera of 41 recovering cholera patients (23 of whom had been immunized against the infection) Rainsford (1952) stated that

"In the non-immunized group it was found that detectable O antibody seldom appeared before the fifth day and the same applied to those of the immunized group who had no detectable O antibody when they first came under observation. In both groups patients were encountered who never produced O antibody in detectable amounts throughout their illness. In the majority of cases that came under treatment early recovery took place several days before O antibody was detected in their sera."

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			H agglutinins	*O agglutinins*
1st	19	0	0	0
2nd	13	0	0	0
3rd	17	0	0	0
4th	8	4	50	12
5th	8	4	50	37
6th	10	5	50	50
7th	13	8	61	46
8th	20	18	90	55
9th	24	18	71	60
10th	23	17	74	57
11th	9	8	90	66
12th	11	9	81	73
Total	175	91	52	

four to nine days after detection of cholera vibrios in their stools, but some times the highest titre was reached already on the day of the first positive findings in the faeces or as late as almost two weeks afterwards Agglutination became negative on the average within 15 days but the reaction could remain positive for 32 days There was no definite correlation between the length of vibrio excretion by the carriers (which varied from one to 21 days with an average of eight days) and the height of the titres but as a rule these were highest in the carriers with the longest excretion periods

(4) *Vaccinated subjects* After early observations by Bertarelli (1905) Karwatzki (1906b) and Serkowski (1906) had shown that cholera vaccination led to a marked agglutination response in the sera of the individuals concerned, this problem was further studied by numerous observers. The following of the earlier findings recorded in this respect deserve particular attention

Balteano & Lupu (1914) concluded from rather limited experiences that

"[*a*] In individuals who had received a single injection, agglutinating power becomes manifest already after 24 hours (1/20) 3 days afterwards this falls to 1/10 to raise again at the end of 48 hours. It reaches a maximum (1/130) after 24 days, then decreases gradually to 1/100 and remains at this level still two months after injection

"[*b*] A similar evolution takes place in individuals who received 2 or 3 injections, except that then the titre reaches 1/150 and remains at this level for 7 days before it begins to decrease."

It has to be added that soldiers in the field who had been vaccinated, retained an agglutinating power of 1:40 for 3½ months. At the end of 5 months their titre was not higher than 1:20-1:30.

Castelli (1917) noted that, while administration of the usual cholera vaccines as well as that of cholera nucleoproteid led to an increase of the bacteriolytic titre in the sera of the vaccinated, an agglutinin response was observable only if the usual vaccines had been administered, but not after nucleoproteid had been given.

Sierakowski (1920a), studying a total of 259 individuals, to whom differently prepared cholera vaccines had been administered, established that differences in the methods of killing these products exerted a marked influence on the agglutinin levels observable in the various groups after vaccination. He further found that (*a*) in three persons tested before and 48 hours and 96 hours, respectively after vaccination, higher agglutination titres became manifest within two days after the vaccine had been given (*b*) in a group of 13 persons the agglutination titres, which had reached values observable in normal persons 6 months after the administration of two vaccine doses, were found to have risen 5-7 days after two booster doses had been given, without, however reaching higher values than after the original two injections and (*c*) 6 months after administration of the booster doses the agglutination titres had sunk once more to levels observable in non-vaccinated individuals.

In the experience of Sano (1921) the agglutination titres in cholera-vaccinated persons reached a maximum in 3 weeks and fell in 10 months to the levels found in non-vaccinated controls.

Hetsch (1928) summarizing the results of these and other observations made during and soon after the First World War stated that though as a rule an agglutinin response was elicited in the sera of cholera vaccinated

Rainsford concluded from these findings and from observations on the duration of the diarrhoea and the length of the vibrio excretion that

"the presence or absence of O antibody had little influence if any on the presence or absence of the vibrio in the stool and was not essential or related to recovery"

The important aim of the investigations of Yacob & Chaudhri (1945) was to establish for how long agglutinins persisted in the sera of cholera convalescents—a subject which in spite of its great importance for a retrospective diagnosis of the disease, had received but little attention in the past owing to the difficulty of observing the convalescents for prolonged periods. Yacob & Chaudhri procured for this purpose sera of persons who had suffered from cholera during epidemics taking place in the three Indian localities in question some months before.

Describing their technique, the two workers stated that they mixed 1/25, 1/50, 1/100 1/150 and 1/200 dilutions of their sera in normal saline in Dreyer agglutination tubes with equal amounts of a suspension of live cholera vibrios, which had been found to react positively with an Inaba O serum. The tubes were kept in the water bath at 56 C for 2 hours and then transferred to a refrigerator readings being taken 24 hours after setting up the test. Results were confirmed by slide-agglutination tests.

The findings made by Yacob & Chaudhri may be summarized as follows

Locality	*Interval between end of outbreak and date of test*	*Number of specimens examined*	*Number of specimens found positive*	*Titres*
Kasur town	about 2 months	11	6	1 50 to 1 150
Narll village	102 days	11	6	1 25 to 1 150
Lahore city	about 3 months	15	1**	1 100

* The interval elapsing between the dates of attack and of serological examination varied from 60 to 64 days.

** Tested 113 days after the time of attack.

Yacob & Chaudhri concluded therefore, "that agglutinins can persist in the blood of recovered cases of cholera up to a period of three-and-a half months and possibly more"—a postulation supported by the observations of some earlier workers, such as Kabelik (1915)

(3) *Healthy carriers* While a number of observers, such as de Bonis (1912) and Sano (1921) found specific agglutinins to be absent or practically absent in the sera of healthy cholera carriers tested by them, positive findings have been recorded in this respect by some other workers, for instance—as has been mentioned above—by Massaglia (1911) and Levi della Vida (1913) and also in a few instances by Shiiba & Oyama (1920) As quoted by Takano Ohtsubo & Inouye (1926) Sakai (1917) found that one-third of the 84 sera of healthy carriers tested by him agglutinated *V cholerae* at titres over 1 200 the highest titre observed in this series being 1 2000 As a rule the individual carriers showed maximal titres from

lower agglutination titres with a maximum of 1:160 in 46 of these individuals, 21 giving negative results.

Observations by Brounst & Maroun (1949) showed that

(*a*) in a group of 371 individuals, inoculated once with 2000 million of an Inaba Ogawa cholera vaccine and tested 30-60 days afterwards with formolized suspensions (2 per 1000) of *V. cholerae* agglutination titres ranging from 1:25 to 1:200 were demonstrable six times only 365 of these sera failing to produce agglutination even at a titre of 1:25

(*b*) 95 of these individuals, who received 50 days after the first vaccination two booster doses totalling 12 000 million *V. cholerae* and whose sera were tested as described above gave similarly disappointing results, agglutination at titres ranging from 1:25 to 1:100 being found only in 11 instances

(*c*) somewhat better results were obtained when suspensions of live cholera vibrios cultivated for 24 hours at 37° C, were used instead of formolized suspensions for the tests the sera of 20 individuals, vaccinated and examined as the persons composing group (*b*) yielded 7 positives (i.e., 35%) the titres ranging from 1:25 to 1:200.

Further comparative tests by Gallut & Brounst (1949) with the sera of 18 individuals, vaccinated and examined as those of the above groups (*b*) and (*c*) confirmed that, except when testing unknown cholera strains with the aid of high-titre rabbit sera, it was not permissible to use formolized *V. cholerae* suspensions for agglutination tests. While obtaining as unfavourable results with the latter suspensions as Brounst & Maroun, Gallut & Brounst recorded 55% and 77% positive results when testing their 18 human sera with live suspensions of an Inaba and an Ogawa strain respectively the titres ranging from 1:20 to 1:500

It will be noted that these findings fully support the conclusion reached in 1916 by Ionesco-Mihaiesti & Ciuca.

(5) *Test vaccination for retrospective cholera diagnosis* At a meeting of the Joint OIHP/WHO Study Group on Cholera held in 1949 Krishnan & Dutta (1950) recorded the following results obtained when administering 1 ml cholera vaccine to the individuals enumerated below and testing the agglutination titre of their sera two weeks afterwards

		Number tested	Agglutination negative	Titres of positive agglutination: 1:20 or less	1:80 or less	1:320 or less	1:1280 or less	1:2560
Normal group	A	18	14	3	1			
	B	16	2	3	10	1		
Inoculated group	A	132	58	31	41	2		
	B	119	6	11	44	58		
Cholera group (convalescents)	A	17			4	9	3	1
	B	4				1	3	

A = *before* test vaccination.
B = *after* test vaccination.

Bearing in mind the scantiness of the evidence available in regard to the group of cholera convalescents the Study Group recommended further observations in order to decide whether this method of test vaccination might be a useful means for the retrospective diagnosis of cholera As was reported at the first session of the WHO Expert Committee on Cholera

individuals, which became maximal after 2-4 weeks and remained manifest for 6-10 months, or according to some workers only for 3-4 months, the titres found varied considerably Hetsch was inclined to ascribe these divergent results on the one hand to variations in the dosage in which the vaccines had been given and to the number of injections administered on the other hand to differences in the technique of the agglutination tests However the observations of Sierakowski (1920) and further experiences referred to below leave no room for doubt that differences in the anti genic value of the various cholera vaccines exert a profound influence on the agglutinin response following their administration. It is certain at the same time that the height of the titres is markedly influenced by differ ences in the agglutination technique Particularly as stressed by Ionesco-Mihaiesti & Ciuca (1916) and confirmed by further experiences, fully satisfactory and therefore comparable, results can be hoped for only when live cholera vibrios and not killed antigens are used for agglutination tests with the sera of the vaccinated.

Recent contributions to the knowledge on the problem presently under review were as follows

Griffitts (1944), to whose observations on the appearance of mouse-protective bodies in the sera of cholera-vaccinated individuals reference will be made later also noted that agglutinins appeared in these sera one week after vaccination, remained at high titre (1 180 to 1 1620) for 1 2 weeks and then diminished, the majority of the sera reacting at low titre 6 months, one year and 18 months after vaccination.

Eisele et al. (1946), testing the sera of (*a*) 7 individuals who had been inoculated twice with a standard cholera vaccine, and (*b*) 27 persons injected twice with different doses of an experimental vaccine which contained mainly the O antigen of *V cholerae* found that specific agglutinins became demonstrable at an equally high titre in both groups and also noted that administration of larger doses of the experimental vaccine did not lead to an increase of the agglutination titres.

Observations made by Gohar & Makkawi (1947) during the 1947 Egyptian outbreak showed that the sera of individuals who had been given a vaccine prepared from the autochthonous Inaba subtype cholera strain, agglutinated this as well as classical Inaba strains to a titre of 1 160 and Ogawa strains to half that titre (1 80)

In the course of interesting investigations, to which further reference will be made below Singer Wei & Hoa (1948b) studied the presence of agglutinins in the sera of 211 cholera-vaccinated individuals 10 days after completion of the immunization. Administration of four different types of vaccines led to a fairly high agglutinin response, the titres often reaching, and sometimes exceeding, 1 320 While the methods of producing these various vaccines did not seem to exert an appreciable effect on the titres which were attained, the agglutinin content of the sera from subjects vaccinated by the intracutaneous route was found to be significantly higher than was the case in the subcutaneously vaccinated individuals.

Ahuja & Singh (1948) to whose observations on 21 persons given 1 ml of cholera vaccine reference has been made before, found that, in marked contrast to the rise of the bactericidal titre in these individuals, the agglutination titres did not become considerably heightened, the maxima reached on the 10th day after vaccination being 1 125 to 1 250 as compared with a pre-vaccination level of 1 25

Erdim (1951), examining the sera of 67 individuals 8 weeks after they had been given two doses of a cholera vaccine containing 8000 million of organisms per ml, found even

agglutinins (*Mitagglutinine*) capable of acting upon heterologous bacterial species The latter, when brought into contact with the agglutinating sera, absorbed only the minor agglutinins, whereas the homologous organisms absorbed the main agglutinins as well Sierakowski emphasized, therefore, the importance of agglutinin-absorption tests for a differentiation of cholera like vibrios showing some serological relationship with *V cholerae* from the latter organisms It has to be stressed, however that in the case of his as well as of Karwatzki s strains equally clear-cut differences from *V cholerae* were revealed by Pfeiffer s reaction.

Observations indicating that organisms belonging to heterogenous bacterial genera may be agglutinated by cholera immune sera seem to have been made first by three German workers during the First World War

Meggendorfer (1918) isolated from the faeces of a healthy soldier a large motile bacillus which was agglutinated to full titre by cholera immune serum but whose homologous serum exerted no action on *V cholerae* Results of agglutination tests with cholera immune serum remained unchanged after the bacillus in question had been subcultivated 74 times. In view of these findings, Meggendorfer stressed the necessity of verifying the vibrio nature of the strains to be serologically tested in cholera-diagnostic work.

As quoted by Meggendorfer Quadflieg (1916) cultivated from the stools of a cholera suspect an *E. coli* strain which, though not reacted upon immediately was after 4 hours agglutinated by cholera-immune serum at titres nearly corresponding to those found in the case of *V cholerae*

Examining roughly 1000 stool-specimens, Messerschmidt (1916) found in about 20—partly in association with cholera vibrios—bacteria belonging to the *E. coli Proteus* or *Sarcina* groups, which became agglutinated at full titre by cholera-immune sera and retained this property after repeated subcultivation, as far as observed for 4 months. Like Meggendorfer he stressed the importance of a preliminary smear examination of the growths to be tested serologically as prescribed in the official German instructions for cholera diagnosis.

Following up an earlier observation by Wong (1936) on two individuals who to treat eye diseases, had been injected intravenously with cholera typhoid vaccine, Wong & Chow (1937)

(*a*) demonstrated the presence of group agglutinins for *V cholerae* and *Brucella abortus* in rabbits immunized by the subcutaneous or the intravenous route with either species or with cholera-typhoid vaccine

(*b*) injecting six human subjects either intravenously or subcutaneously with this vaccine, found that while the sera of all six individuals agglutinated cholera vibrios as titres of maximally 1 160 4 of the sera also agglutinated *Br abortus* at titres of maximally 1 40

The validity of these initial findings was fully confirmed through observations on larger groups of cholera vaccinated individuals by Eisele and colleagues (1946 1947 1948) and by Erdim (1951) That the brucella agglutinins developing in the sera of such persons—often at higher titres than observed by Wong & Chow—are apt to persist for considerable periods is well illustrated by the records of Eisele et al (1947) who demonstrated the presence of these agglutinins in 27% of the members of a group tested 18 to 28 months after cholera vaccination

in 1951 further investigations had not confirmed the usefulness of the above method for the retrospective diagnosis of individual cholera cases. It was considered possible, however that the procedure might be helpful in establishing the nature of infection in *groups* of people who giving a history of diarrhoea and vomiting, were suspected of having recently suffered from cholera The conclusion arrived at by the Committee was

"that the method thus far had not given results of definite practical value. Should work in this direction be continued, attention ought to be given to the possible role of cross-reactions due to the presence of brucella or salmonella infections. For this reason and on general grounds great attention ought to be paid to the advisability of using in addition vibriocidal tests for the retrospective diagnosis of cholera." [Page 5]

The usefulness of the method of test vaccination for the retrospective diagnosis of cholera in groups of suspects was recently upheld by Lahiri & Dutta (1954) who drew attention to the following figures

		Number tested	Percentage negative	Percentage positive at 1 160 or less	Percentage positive at 1 320 or above
Bacteriologically confirmed cholera cases	A	94	2	46	52
	B	29	0	28	72
Clinically diagnosed cholera cases	A	26	15	50	35
	B	10	0	50	50

A = *before* test vaccination.
B = *after* test vaccination.

(C) Paragglutination and co-agglutination

As summarized by Sierakowski (1920b) in an important study devoted to the problem of *Mitagglutination* in cholera, Karwatzki (1906a) reported that he had met with some cholera like vibrios which agglutinated at fairly high titres with cholera immune serum, but could be distinguished from *V cholerae* with the aid of Pfeiffer s reaction—results which are not surprising when the inadequacy of the sera then available for agglutination tests is considered.

Sierakowski himself found among the numerous vibrio strains isolated by him during the 1914-15 cholera outbreak in Galicia and Poland six strains of water vibrios and one derived from the faeces of a cholera convalescent, which showed morphological, cultural, and biochemical properties compatible with those of *V cholerae* and agglutinated at fairly high titres with sera obtained by cholera immunization of horses, in two instances also with specific rabbit sera. However, while one of these seven strains reacted like *V cholerae* in complement-fixation tests, none gave a specific Pfeiffer reaction. Moreover the sera raised against the seven strains did not agglutinate cholera vibrios and, while the latter were found to be capable of absorbing the agglutinins for the seven strains from cholera immune sera, the reverse did not hold true, the suspect vibrios leaving the cholera agglutinins intact, if allowed to absorb cholera-immune sera.

Trying to interpret these findings, Sierakowski postulated that the agglutinating sera contained besides main agglutinins a series of minor

In their valuable study on the laboratory aspects of the 1947 cholera outbreak in Egypt, Gohar & Makkawi (1948) noted that on several occasions suspicious colonies which gave positive results in slide agglutination tests with a cholera O serum were ultimately identified as *B faecalis alcaligenes* Following up these observations, Gohar & Makkawi confirmed not only that *B faecalis alcaligenes* strains were agglutinated at a titre of 1 25 by cholera O serum but established also that *V cholerae* was reacted upon at the same titre by immune sera raised against *faecalis alcaligenes* strains However when absorption tests were made neither these organisms nor cholera vibrios were found capable of completely absorbing the agglutinins from the heterologous sera It appeared, therefore, that the organisms concerned "*merely shared an O-antigenic fraction*" Gohar & Makkawi also referred to previous observations by one of them which had shown that *V cholerae* shared an H antigenic fraction with *Salmonella enteritidis*

A further study of the antigenic relationship existing between the Inaba strains of *V cholerae* isolated in Egypt and *S enteritidis* was made by Felsenfeld (1948) He concluded from agglutination and agglutinin absorption tests made with 3 Egyptian cholera strains and various *Salmonella* strains, respectively with Inaba H O and *Salmonella* O and H sera, that the *V cholerae* strains contained fractions of the *Salmonella* antigens I XII and g.

A more general investigation of the serological relations existing between *V cholerae* and common Enterobacteriaceae was undertaken by Felsenfeld et al. (1951) Making slide agglutination tests with cholera H O sera they obtained the following results with various faecal strains isolated at Chicago

Organism	*Number tested*	*Number positive in agglutination tests with H-O sera raised against V cholerae* Inaba	Ogawa
E. coli	70	11	2
A aerogenes	134	26	1
Paracolobactrum	5	1	0
Proteus	29	2	0
Pseudomonas	55	2	0
Faecalis alcaligenes	6	0	0
Streptococcus	16	7	2
Total	315	49	5

Absorbing Inaba H-O serum with *Brucella melitensis Br suis S enteritidis E coli A aerogenes* and *P morgani* strains, Felsenfeld and co-workers found

"a fraction of the cholera H antigen to be identical with the factor common to the g.m. antigen of Salmonellae and a part of the Brucella flagellar antigen, while a common O antigen component was found to be part of the I IX. XII antigen of Salmonellae"

Following up and amplifying laboratory studies by Eisele et al (1946) McCullough, Eisele & Beal (1948) reported that

"Reciprocal agglutinin-absorption tests on antiserums for *V comma* and the brucella species (*abortus suis melitensis*) showed conclusively that the antigen shared by these groups of organisms is an H antigen of *Vibrio comma*. This antigen is present in all three species of brucella with minor qualitative and quantitive differences."

In strict contrast to this conclusion Gallut (1950) stated that the cholera vibrio and the brucella species possessed common O antigens found to correspond to the antigenic factors C and D of *V cholerae* for the strain of *Br suis* tested, and to the factor D only in case of the two *Br melitensis* strains examined Recording the observations he made when testing these brucella strains with cholera immune sera Gallut noted that

"It is known that for cholera diagnosis agglutination tests must be made with unheated vibrio suspensions, preferably those of living vibrios [Gallut & Brounst, 1949]. By this technique the El Tor vibrios cannot be differentiated from the authentic cholera vibrios. However their different chemical composition (protein I of Linton for the *V cholerae*, protein II for *V El Tor*) produces differences in their serological behaviour after heating for 3 hours at 56°C. We submitted *Br abortus* suspensions to such heating and found that these organisms behave like true cholera vibrios and not like El Tor vibrios This has been confirmed by the use of a serum raised against the protein of *V El Tor* which failed to agglutinate *Br abortus suis*" [Trans]

In a further paper Gallut (1953c) stated that he had studied the question whether a pure A type cholera immune serum could be obtained by the absorption of a serum raised against the Inaba variant of *V cholerae* (which according to him had the antigenic formula A C) with *Br suis* It was found that the latter organisms were incapable of fully absorbing the C antibody of Inaba serum.

As Gallut (1954b) added in a subsequent paper devoted to a critical evaluation of the retrospective serodiagnosis of cholera, he had been able to make the following observations on an individual who while never having been vaccinated against that disease, had shown signs of a brucella infection contracted in the laboratory in October 1953

Species used for agglutination test	*Maximum titres*	
	10 October 1953	*6 January 1954*
Br abortus suis	1 1666	1 80
Br melitensis	1 2000	1 80
V cholerae Inaba	1 1000	1 80
V cholerae Ogawa	1 100 (partial)	0

Commenting on these findings, Gallut stated

"Thus even after three months the cholera agglutination titre was significant. It is evident that such a serodiagnosis, if made with a vibrio of the Inaba variety indicates a retrospective diagnosis of cholera. Therefore, since the common factor is absent from the endemic Ogawa variety (AB), it seems judicious and essential to us to utilize solely suspensions of the latter variety for the serodiagnosis of cholera in cases where this is associated with a positive serodiagnosis of brucellosis." [Trans]

with the strains and even with the diagnostic serum of the Russian observers, were unable to confirm the findings of the latter Stamm (1914) found that several of the cholera strains tested by him no longer became agglutinated with cholera immune serum after they had been repeatedly passed through water, while others remained unaltered even after more frequent passages The strains which were no longer agglutinated, were almost without ex ception still immunogenic i e. capable of producing immune sera positively reacting with cholera vibrios. Since in Stamm s opinion variations of *V cholerae*, including changes in agglutinability were not easily effected under natural conditions but, once they had taken place, were apt to be permanent, he declared that

" consequently it is impossible to explain the rise, cessation and recrudescence of cholera epidemics through the hypothesis of a transmutation of the cholera vibrios into saprophytic variants and *vice versa* " [Trans.]

Similar views were vigorously expressed by the orthodox German school, Kolle (1909b) even maintaining that any "non agglutinable" vibrio found in human stools was not a cholera vibrio It has to be noted, however that this extreme view was reached at a time when the phenomena of dissociation were still unknown Indeed it would seem that Finkelstein (1931) was the first worker inclined to ascribe the loss of agglutinability by *V cholerae* after a sojourn in water to a transition from the smooth into the rough state.

Finding that, if cholera stools were added to Indian tanks (water re servoirs) *V cholerae* invariably lost its agglutinability with specific serum within 16-20 hours Tomb & Maitra (1926) were led to consider the "agglutinating" faecal vibrios and the "non-agglutinating" organisms met with in the tanks, and also in human carriers, as identical in character The far reaching conclusions these two workers drew in strict contrast to Stamm s views from these and related observations will be discussed later on.

Brahmachari (1927 1928 1929) to whose work also further reference will be made below maintained like Tomb & Maitra that passage through water rendered the cholera vibrios inagglutinable with specific serum and also claimed that intravenous injection of rabbits with *V cholerae* led to the appearance of inagglutinable vibrios in the stools of the animals—a result which in view of the ubiquity of cholera like vibrios in India ought to be considered as a *post hoc* rather than a *propter hoc* phenomenon.

Minervin (1931) in order to study the changes *V cholerae* was apt to undergo when introduced into specifically immunized animals, injected typical cholera vibrios into the testicles of cholera immune rabbits and excised these organs 3-10 days later In three instances it was possible to isolate from the excised testicles cultures of vibrios which had obviously undergone roughening. Agglutination tests with these passage strains gave at first negative results, but it was possible to restore agglutinability in one

Further testing the agglutinability of various *Paracolobactrum* species with cholera-immune sera, Malizia (1954) recorded the following noteworthy results

		Agglutination with					
		H-O cholera sera			*Absorbed (O) cholera sera*		
Antigens from	*Strains tested*	*Inaba and Ogawa*	*Inaba serum only*	*Ogawa serum only*	*Strains tested*	*Inaba*	*Ogawa*
P coliforme	120	4	3	8	7	0	0
P intermedium	10	0	0	4	2	0	0
P Bethesda	10	1	4	1	4	1	0

As Malizia stated, these findings indicated

"a fairly close antigenic relationship between the *P Bethesda* strain and the *V comma* (Inaba) strain. Whether this similarity indicates that this strain plays a more important role in enteric infections than previously recognized warrants further study"

Changes in agglutinability

In order to do full justice to the problem of whether agglutinability by the usually available specific sera is an unalterable characteristic of *V cholerae* permanently distinguishing this organism from the cholera like vibrios, it is necessary to scrutinize not only whether authentic cholera vibrios may lose their agglutinability by the above-mentioned sera, but also whether agglutinability by such sera may be acquired by vibrios initially giving negative reactions in this respect.

Loss of agglutinability Apart from Bordet, who claimed in 1896 that animal passage could render the cholera vibrio inagglutinable with specific serum, Ransom & Kitashima (1898) seem to have been the first workers to have devoted attention to the subject presently under review They noted that, in contrast to its agar grown parent strain, a substrain of *V cholerae* which had been passed twenty times through broth containing 1% cholera immune serum, exhibited feeble or even no agglutinating power if incubated for 24 hours in 10-ml amounts of the same medium with a specific serum content of 1%-2%.

The original observation of Ransom & Kitashima that growth in the presence of its homologous serum is apt to impair or to inhibit the agglutinability of *V cholerae* has been repeatedly confirmed by subsequent observers. There can be no doubt that a transition into the rough state is responsible for this well-authenticated alteration.

Some workers in Russia, particularly Zlatogoroff (1909 1911) and Horowitz (1911) seem to have been the first to assert that *V cholerae* was apt to lose its specific agglutinability in the human intestine or in water supplies and a few other observers such as Barrenscheen (1909) and Puntoni (1913b) confirmed the findings made in the latter respect by Zlatogoroff. However several other investigators such as Haendel & Woithe (1910) Köhlisch (1910) Wankel (1912) and Bindi (1913) though working in part

vibrio strains, originally found to give negative results in agglutination tests, agglutinable with cholera immune serum The methods he used for this purpose were (*a*) frequently repeated subcultivation, resulting in the appearance of agglutinable vibrios in the 54th generation, (*b*) subcultivation alternating with intraperitoneal passage of the strains through guinea pigs and (*c*) combination of the latter procedure with the simultaneous injection of killed typhoid or *E. coli* cultures or of living streptococci so as to increase the virulence of the growths Evaluating his results, which he stated that he had confirmed with the aid of Pfeiffer's reaction in one instance and through complement fixation tests eight times Zlatogoroff pointed to the importance of the water vibrios as a potential source of cholera infection

Reporting on further investigations, Zlatogoroff (1911) stated that he had been successful in restoring the agglutinability of *V cholerae* lost in the human intestine not only with the aid of the above mentioned methods, but sometimes also by subjecting the serologically altered vibrios after they had been suspended in cholera immune serum diluted with normal horse serum, to repeated freezing and thawing. The method of simply passing the vibrios through diluted cholera immune serum seemed unreliable because of the possibility of its leading to spontaneous agglutination

Zlatogoroff concluded from his observations that

"each vibrio which is isolated from the faeces during an epidemic or at its onset, even if it does not agglutinate, should cause suspicion of cholera for the reason that the agglutinability of the cholera vibrios is very changeable" [Trans.]

Horowitz (1911) who found besides intense subcultivation symbiosis with *Sarcina lutea* effective in restoring the agglutinability of *V cholerae* lost in the human intestine, reached an identical conclusion [1]

Several other workers, such as Köhlisch (1910) McLaughlin & Whitmore (1910) Freifeld (1912) and Wankel (1912) were not able to confirm the validity of the above recorded findings, the last mentioned observer stating that

"even though the techniques recommended by Horowitz and Zlatogoroff have been followed most painstakingly it was not possible to transmute even a single of the ten strains from Petersburg into an authentic cholera strain" [Trans.]

A further noteworthy observation made by Douglas (1921) concerned a "paracholera" vibrio strain which after repeated subculture on artificial media had acquired agglutinability with cholera immune serum However, since suspensions of this organism were found incapable of absorbing the agglutinins for *V cholerae* from the immune serum, the positive reactions it gave in agglutination tests were apparently not of a specific nature. It served as a corollary for this assumption that a serum raised against the paracholera vibrio failed to agglutinate *V cholerae* even at a titre of 1 100

[1] It should be noted that Pentoni (1913a) also claimed good success when growing a strain of "inagglutinable" vibrios five times in succession in the presence of two organisms isolated from the air

instance through repeated subcultivation, and in the other two through animal passage (intravenous injection of rabbits)

D Hérelle, Malone & Lahiri (1930) observed some instances of inagglutinability with specific immune serum in the case of cholera vibrios isolated from the faeces of convalescents and suggested that this change was the result of bacteriophage action Referring to these observations, Vardon (1940) expressed the opinion that the corresponding changes noted by Tomb & Maitra (1926) in the case of faecal vibrios exhibited in water tanks might have been due to the same cause. Morison (1932) while admitting that " the bacteriophage has something to do with the production of rough from smooth vibrios and *vice versa* " added that he had " been unable in the case of cholera to make agglutinating vibrios inagglutinable by growing them in the presence of bacteriophage " However Doorenbos (1932) adduced evidence to show that the presence of bacteriophage was apt to alter the agglutinability of cholera vibrios and it would seem that Morison (1935) obtained the same result when using instead of pure-line bacteriophage-strains combinations of different races

Yang (1935) keeping cholera vibrios derived from one strain in the dark at room temperature in raw candle-filtered and autoclaved water samples respectively found that the agglutinability of the organisms became lost in the raw and filtered river water samples as well as in the raw canal and well water samples after periods ranging from 21 to 28 days.

The present writer on the contrary when studying the survival of cholera vibrios in numerous filtered and autoclaved samples of Shanghai surface waters kept under conditions identical to those used by Yang, was never able to observe a loss of agglutinability of the test organisms, even though some of the specimens could be watched for a period of almost a year Vibrios which were not agglutinable with cholera-diagnostic sera could be isolated not rarely side by side with *V cholerae* from the stools of patients. Since, however cholera like vibrios abounded in the surface waters and were occasionally met with in the stools of healthy individuals at times when cholera was absent from Shanghai, it was not possible to consider the occurrence of " inagglutinable " organisms in the cholera stools as significant. This was in accord with previous observations by Crendiropoulo (1912) who adduced evidence to show that the apparent replacement of *V cholerae* by inagglutinable organisms in the stools of carriers was really the result of an initial co-existence of cholera and cholera like vibrios in the intestines of these individuals.

(2) *Acquisition of agglutinability* Zlatogoroff, apparently the first worker who paid systematic attention to the subject presently under review to which some general reference had already been made by Sanarelli (1893) claimed in 1909 that he had succeeded in rendering 10 out of 18 water

vibrio strains, originally found to give negative results in agglutination tests, agglutinable with cholera immune serum The methods he used for this purpose were (*a*) frequently repeated subcultivation, resulting in the appearance of agglutinable vibrios in the 54th generation (*b*) subcultivation alternating with intraperitoneal passage of the strains through guinea pigs and (*c*) combination of the latter procedure with the simultaneous injection of killed typhoid or *E coli* cultures or of living streptococci so as to increase the virulence of the growths Evaluating his results, which he stated that he had confirmed with the aid of Pfeiffer s reaction in one instance and through complement-fixation tests eight times Zlatogoroff pointed to the importance of the water vibrios as a potential source of cholera infection

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[1] It should be noted that Puntoni (1913a) also claimed good success when growing a strain of inagglutinable vibrios five times in succession in the presence of two organisms isolated from the air.

These findings well illustrate that claims regarding the appearance of agglu tinability in vibrios have to be interpreted with great caution, unless they have been supported by further and thorough serological examinations of the supposedly transmuted strains

Tomb & Maitra (1927 1928) though admitting that the few attempts they had made in the laboratory to render initially inagglutinable vibrios agglutinable with cholera immune sera had given " inconclusive and in constant " results, laid great stress upon the fact that they had invariably been able to effect a transmutation of cholera vibrios into an inagglutinable form in their water tank experiments and also upon observations regarding the frequency of organisms of the latter type in human faeces as well as in the tanks. They felt entitled to conclude from this evidence

" that the non-agglutinating vibrio takes on the agglutinating characteristic under certain biochemico-physical conditions in the human intestine the nature of which are at present unknown, and in this mutation or epidemic form is the cause of epidemic cholera, since it is not unreasonable to assume that a characteristic so unstable may be as easily acquired as lost "

Claims as fargoing in their implications as those of Tomb & Maitra were made by Brahmachari (1927 1928 1929) and by Pasricha and col leagues (1931 1933)

The former worker reported in 1927 that not less than 40 out of 68 strains of vibrios which had been isolated in an endemic area in Calcutta from patients showing clinical signs of cholera, healthy persons, or water tanks, and which initially did not become agglutinated with cholera immune serum gave positive results in agglutination tests after they had been kept for six months, particularly if they had been frequently subcultivated.

Brahmachari (1929) also claimed that he had been able to restore through animal passage the agglutinability of cholera vibrios which had become spontaneously lost in the intestine of an intravenously infected guinea pig.

Testing the action of cholera bacteriophages on 355 cholera-like strains, Pasricha, De Monte & Gupta (1931) found that " the secondary 'phage-resistant colonies that develop after the action of cholera bacteriophages in some experiments are agglutinable by cholera high-titre serum. They completely absorb the agglutinins from a cholera-type serum and produce a serum which agglutinates cholera vibrios in very high dilutions "

Pasricha and colleagues admitted that this acquired agglutinability was difficult to maintain on subculture, requiring repeated plating and selection of the best agglutinating colonies. They felt certain, nevertheless, that a large proportion of the vibrios found in cholera affected places, which differed from *V cholerae* only in their serological reactions, were mutant forms of the latter organism and played " a great part in the aetiology of the disease "

In their second paper (1933) Pasricha, De Monte & Gupta maintained that an action identical with that of the cholera phages was exerted by *vibriophages* which were capable of lysing only cholera like but not true cholera vibrios. They claimed in this connexion to have obtained the following results from experimenting with 56 strains of recently cholera-like vibrios

Remained serologically unchanged after bacteriophage action	32
Became agglutinable after action of *cholera* phage	11
Became agglutinable after action of *vibriophage*	13
Total	56

Yang (1935) subjecting four water vibrio strains which were originally inagglutinable with H + O cholera immune serum, to daily subculture in plain broth and sheep-serum broth respectively, found that all samples except one subcultivated in sheep-serum broth became rather suddenly agglutinable with the specific serum after intervals ranging from two to six days.

Summarizing the results of a study on vibrios isolated from non-cholera sources in India Taylor & Ahuja (1935a) stated that

"A vibrio isolated from water in an area widely removed from places where cholera is endemic and which had been free from cholera for a number of years, was inagglutinable when first received, but in a period of six months sub-culture in the laboratory developed all the biological characters of an authentic cholera vibrio including H and O agglutination to full titre, and was indistinguishable from a cholera strain when quantitative and qualitative tests were applied."

While stressing that this strain differed in chemical structure from typical strains of *V cholerae* Taylor & Ahuja stated that the epidemiological significance of vibrios agglutinable with cholera immune serum, yet chemically differing from typical Group I cholera vibrios, was not clear it having not yet been determined whether the vibrios of an aberrant chemical structure might be cholerigenic.

In a second paper (1935b) Taylor & Ahuja stated that they had been able to produce through a series of intraperitoneal passages in mice agglutinable variants giving the H and O reactions of typical *V cholerae* strains in the case of three formerly inagglutinable vibrios namely (1) a strain of *V metchnikovi* (2) a water vibrio isolated three years previously in Calcutta and (3) a vibrio obtained in an endemic area of Bengal from a healthy person In the case of the two last mentioned strains the acquisition of agglutinability was accompanied by a shift in chemical const tution.

Aptly summarizing observations in point made by Linton, Shrivastava & Mitra (1935 see also Linton, 1935) White (1937b) stated that

"From a first plating of cholera stool two colonies were picked off yielding, respectively a typical culture of *V cholerae* termed Rangoon smooth and a vibrio race, termed Rangoon rough 1 held to be a rough derivative of *V cholerae* and showing no serological nor antigenic relationship with that organism From Rangoon rough 1 there was isolated a race, Rangoon rough 2, growing in convoluted colonies and serologically distinct from Rangoon smooth and Rangoon rough 1 Next there was separated from Rangoon rough 2 a fourth race, Rangoon rough 2a, described as smooth-rough, serologically intermediate between Rangoon smooth and Rangoon rough 2 and finally from this a fifth race Rangoon smooth recovered in which the distinctive serology of *V cholerae* was completely restored."

These shifts in agglutinability were stated to have been accompanied by shifts in the chemical composition of the successively isolated strains while " Rangoon smooth " and " Rangoon rough 1 " were found to belong to the chemical Group I of Linton and colleagues " Rangoon rough 2 "

showed the chemical characteristics of Group V, and "Rangoon smooth recovered" those of Group VI

Commenting upon these modifications, Linton (1935) declared that either they might have resulted from successive changes in the molecular arrangement of the vibrio proteins and carbohydrates, or all the different variants might have been present in the originally isolated strain. However in a further paper Linton, Seal & Mitra (1938) stated that they had obtained from a strain of "Rangoon rough 2," cultivated from a single cell, through 10 daily transfers in 0.5% glucose broth a variant which was inagglutinable with "Rangoon rough 2" immune serum, but was agglutinable with the serum raised against the original smooth Rangoon strain and was otherwise as well indistinguishable from the latter showing the chemical constitution of Group I. The serological results obtained with H + O sera were confirmed through agglutination tests with an Inaba O serum which, while not producing a reaction with the "Rangoon rough 2" strain agglutinated the smooth variant obtained from this at titres up to 1 2500

Discussing these findings, Linton and co-authors stated that

"Although almost nothing is known about the internal arrangement of the vibrios, it may perhaps be permissible to suggest that each of them possesses the enzymic equipment capable of synthesizing the various proteins and polysaccharides which are found in the whole group."

Yu (1940) recorded that he had been able to render 16 out of 20 water vibrio strains, which had been isolated at the time of a cholera outbreak in Shanghai from the Whangpoo river partially agglutinable with cholera O serum by passing the organisms suspended in mucin five times in succession by the intraperitoneal route directly from guinea pig to guinea pig. He felt entitled to conclude from this observation that such transformations might also take place under the influence of mucin in the human intestine particularly in the case of gastro-intestinal disturbances, when mucoid substances were apt to be abundant. It has to be emphasized, however that Yu's strains had initially shown a trace of agglutinability with cholera O serum. Hence as maintained by Gallut (1951) these organisms possibly contained minor O-antigenic factors apt to react with non-specific components of the O serum used. Be this as it may Gallut (1951) obtained entirely negative results when repeating Yu's experiments with seven Egyptian water vibrio strains, which were apparently similar in their initial serological properties to the strains of the latter worker While Gallut's strains did not acquire any specific agglutinability when being passed in mucin suspension ten times from mouse to mouse, they lost after the third to the sixth passage the agglutinability with their homologous serum, so that mucin seemed to degrade rather than to enhance the serological properties of the organisms. Some modifications of the chemical properties became noticeable in the course of the passages but these occurred also in the vibrios passed

in mucin free suspensions through control animals and even in the cultures of the strains kept in stock.

As will be perceived from above recorded statements, numerous workers have claimed success in restoring the lost specific agglutinability of cholera vibrios or even in transmuting cholera like vibrios, which originally failed to react positively in agglutination tests with cholera immune sera into organisms behaving in this repect like *V cholerae* However, in view of the technique adopted by them, the results recorded by most of these workers have to be viewed with great scepticism. They often failed to confirm their findings through adequate agglutinin-absorption and cross-agglutination tests. Further, it is frequently impossible to rule out the possibility that the strains with which the experiments were started were not of a uniform composition but contained besides a large number of organisms reacting negatively in specific agglutination tests an initially unrecognized minority of true cholera vibrios Most important finally it must be kept in mind that with a very few exceptions the observations referred to above have been made with not fully specific H + O sera

It is under these circumstances not surprising to find that, whenever some workers reported that they had brought about a serological transmutation of vibrios others even though using identical methods, failed to substantiate these claims It has been stated already in this connexion that several investigators repeating the experiments of Zlatogoroff and of Horowitz, were unable to confirm the findings of these two observers. It has likewise been noted that, history repeating itself Gallut (1951) recently obtained strictly negative results when checking the validity of claims similar to those of Zlatogoroff and Horowitz, made by Yu in 1940 The papers read by Tomb & Maitra and by Brahmachari in 1927 were also much criticized, Pandit, for instance, stating that, though he had kept cholera like vibrios isolated from water supplies in India for over two years, he had failed to note the change in the agglutinability of such strains claimed to be frequent by Brahmachari

A most determined stand against what he called the " legend " of serological transmutability of vibrios was taken by White (1937b) Discussing in particular the above mentioned observations of Linton and colleagues White maintained that since

> " Rangoon smooth and Rangoon rough 1 were derived from two colonies in a first plating of stool, belief in their genetic connection is a matter of pure assumption "

White s main objection not only to the validity of the claims made by Linton and co-authors but also to that of the results recorded by Taylor & Ahuja (1935b) was based upon tests he made with a special type of cholera bacteriophage the LL phage (White 1937a) He stressed that both in the case of the Rangoon series and in that of Taylor & Ahuja s strains, cultures found to be infected with this phage were alleged to have been derived from

growths in which it was absent. Discussing this discrepancy White said that

"Various hypotheses may be improvised to fit the facts genesis of bacteriophage *de novo* mutation of vibrio phage or vibrio phages unknown, collaterally with that of the vibrio itself but the simple and obvious indication is that the alleged mutant cultures are not derived from the parents presented."

Generally speaking, White maintained that

"There is, I believe, not only insufficient evidence on which to base a theory of vibrionic transmutability such as is at present current, but definite evidence against acceptance of such alleged instances of change as have been discussed."

Referring again to the Rangoon strains, White (1940a) stated that

"The negative results obtained with extracts of a rugose derivative of Rangoon rough 1 which is a smooth culture with the serology of *V metchnikovi*, and of the capsulated culture Rangoon rough 2 support my contention that these strains have no immediate relation to nor derivation from their alleged parent, the cholera strain Rangoon smooth."

In view of White s objections it is difficult to assert the validity even of Taylor & Ahuja s observa ions However even if one could admit the possibility that under highly artificial conditions the agglutinatory properties of vibrios might become changed, there is no convincing evidence to show that such transmutations take place under natural conditions and that consequently cholera like vibrios or cholera vibrios which had lost their agglutinability with the usual specific sera form a reservoir from which epidemics may be produced *de novo* It is significant to note that Seal (1935) one of Linton s principal co-workers, discussing in particular the variations brought about by bacteriophage in the laboratory considered it an open question whether such changes in the character of vibrios "do also occur in nature or inside the human system"

A peremptory statement made in this respect by d Hérelle (1928) when discussing Tomb & Maitra s claims, was that he could

"not agree with the possibility of the regression from non-agglutinating to agglutinating In our quarantine station of Tor during the last fifty years, hundreds of thousands of pilgrims harbouring non-agglutinating vibrios in their intestine have passed through the station on their way towards the North, and not a *single* case of cholera has been discovered amongst them nor has an outbreak of cholera ever occurred north of Tor We must conclude that, in Nature, the regression from non-agglutinating to agglutinating vibrios does not take place and that carriers of such non-agglutinating vibrios are harmless and never the origin of an outbreak of cholera. To say that non-agglutinating vibrios may be the cause of the epidemicity is a mere hypothesis, but to show that a Mecca pilgrim carrier of non-agglutinating vibrios has never been the cause of an epidemic, that is a fact."

Gallut (1951) commenting on the significance of Yu s claims, similarly stressed that observations on the incidence of cholera in Shanghai did not support the idea of a causative role played by the water vibrios in the origin of the epidemics.

The validity of this statement is fully supported by the observations the present writer had opportunities to make in most cholera affected parts of China during about a score of years Though water vibrios were found to abound everywhere not a single cholera outbreak was seen which was not found to have been due either to an importation of the infection or to its continued sporadic occurrence in man In this sense therefore one is certainly entitled to consider the alleged serological transmutability of vibrios to be a myth.

Haemo-agglutination

As summarized by Doorenbos (1932) he observed in 1931 that, when a few drops of a suspension of sheep erythrocytes were added to a saline suspension of recently isolated El Tor vibrios after a few minutes the colour of the blood changed to violet and at the same time the blood corpuscles began to become agglutinated in the form of small flocculi which rapidly sank to the bottom of the tubes. Further studying this phenomenon Doorenbos established that

(*a*) the reaction took place at 37 C as well as at 0°

(*b*) the haemo-agglutinins were inactivated by heating the suspensions for 5 minutes at 64 C

(*c*) the haemo-agglutinins were absorbed by red blood-corpuscles

(*d*) some strains possessed the property of haemo-agglutination only during a short period of their development the phenomenon suddenly appearing after a few hours incubation and disappearing as quickly when the cultures became older

(*e*) the haemo-agglutinins inhibited the action of the haemolysins and vice versa.

Doorenbos added that seven Syrian strains, recently isolated from carriers who had arrived from a cholera affected area (Iraq), produced a marked *haemo-agglutination only in guinea pig blood suspensions*, and a feeble reaction if goat blood was used Haemolysis tests with these strains gave more clear-cut results proving positive with guinea pig blood and negative with goat blood

Panayotatou (1931) using guinea pig blood for such tests, had satisfactory results with four strains from Basra, haemo-agglutination becoming manifest before haemolysis became apparent. She recommended using for the former tests 4- to 6-hour-old cultures suspending the vibrios in broth diluted 1/10 1/100 and 1/1000 respectively with normal saline and reading the results after an incubation at 37 C for 15 minutes. She confirmed that haemo-agglutination disappeared as soon as haematolysis set in.

While in the opinion of Panayotatou haemo agglutination tests appeared to be of value for the laboratory diagnosis of cholera, Cantacuzène (1933) finding that identical reactions were given by various cholera like vibrios, considered such tests to be without practical importance

Gallut & Brumpt (1944) explored the possibility of whether in cholera diagnostic work advantage might be taken of the method of "haemo-agglutination" of Brumpt (1941) for which, instead of the serum, the whole blood of the patients was used for rapid tests on slides or gelatinized paper. Besides working with formolized H+O suspensions, Gallut & Brumpt also made tests with O suspensions of *V. cholerae* prepared by suspending alcohol killed vibrios after centrifugation in 10% sodium citrate solution and adding one drop of a 1% methylene blue solution per ml. The rabbits, whose blood was tested, had been immunized either with H+O or with O antigens. It was found that O as well as H+O haemo-agglutination took place within a few minutes or even seconds at ordinary temperature while tests with the blood of normal animals or of healthy human beings gave negative results. Gallut & Brumpt therefore recommended this method which had proved satisfactory in the case of other infections, such as typhoid, paratyphoid, and typhus, for the diagnosis of cholera. So far however no practical advantage seems to have been taken of this recommendation or of the proposal of Felsenfeld and co-workers (1955) to utilize for the purpose of cholera diagnosis the haemagglutination method suggested by Neter and colleagues (1952) for the recognition of enteric infections producing low serum agglutination titres.

Acid agglutination

It being proposed for the convenience of the record, to deal with acid agglutination tests at the present juncture it has first to be noted that Beniasch (1912) and Sgalitzer (1914) found this method unsuitable for a differentiation of cholera and cholera like vibrios. However Vercellana (1926) recorded that, when tested with lactic acid cholera like vibrios became invariably and rapidly agglutinated in the form of large and stable flocculi whereas cholera vibrios if reacting at all, became agglutinated at lower dilutions only in the form of small and unstable flocculi. Dambovіceanu (1933) testing 63 strains of *V. cholerae* and 34 cholera like strains with the aid of acid agglutination tests found that more than half (58%) of the latter reacted like the cholera vibrios. However, she expressed the opinion that tests of this kind might be of value for a distinction of cholera like strains which were descendants of *V. cholerae* from those having no genetic relation with this organism.

Precipitin reactions

Though, as noted above Kraus (1897) drew attention early to the precipitin reactions taking place when filtrates or extracts of cholera cultures were brought in contact with specific immune sera, this method was not adopted for the purposes of routine laboratory diagnosis in view of the close correspondence found to exist between the results it gave and those

obtainable with the more expedient agglutination tests [1] Observations proving this rule have been made under various conditions for instance, by Balteano & Lupu (1914) when using the sera of cholera vaccinated individuals for parallel agglutination and precipitin tests, and by Dambovíceanu et al (1934) who applied both these methods to study the immunogenic properties of the residual antigen extracted from *V cholerae* with the aid of trichloracetic acid. It is also noteworthy that Gallut (1950), making precipitin tests with the glucolipidic extracts of *Br suis* and cholera immune sera on the one hand with the corresponding extracts of cholera vibrios and various anti brucella sera on the other, obtained results identical with those of analogous agglutination tests. However Shrivastava & Seal (1937, see also Linton, Seal & Mitra, 1938) recorded that by making precipitation reactions with the vibrio polysaccharides isolated by them and various immune sera, it was possible to distinguish between cholera and El Tor vibrios which though behaving identically in O agglutination tests, showed a different chemical constitution

Shrivastava & Seal (1937) determined the precipitin reactions given by polysaccharides isolated from (1) a Group I Inaba strain of *V cholerae* and (2) an Inaba variant belonging to the chemical Group VI of Linton and collaborators with the aid of immunne sera representative of each of the six groups into which the vibrios were divisible on the basis of their chemical constitution. It was found that in the case of the typical Inaba strain, positive reactions were obtained only with Group I immune sera, while the Inaba variant reacted only with the two Group VI immune sera used. Three sera raised against El Tor vibrios falling into the chemical Groups IV or V failed to react with either of the two above-mentioned polysaccharides.

Continuing these studies with polysaccharides prepared from 23 vibrio strains belonging to different chemical groups, the haemolytic properties of which were not stated, Linton, Seal & Mitra (1938) reached the general conclusion that

" precipitin reactions between the polysaccharides and antisera to the whole organisms indicate that in general the serology expresses the underlying chemical pattern of these organisms and indicates the same groups as the chemical analysis "

It also deserves attention that Gallut & Grabar (1943a) noted the presence of marked differences even among vibrios belonging to one and the same serological group when quantitatively assessing with the aid of nitrogen determinations the precipitations which resulted from the action of fixed amounts of various immune sera upon variable amounts of the glucolipidic antigens of the organisms. They suggested that application of this method might be of value in defining the serological characteristics of the vibrios.

A profound study of the serological properties of *V kadiköj* a " haemotoxic " (haemolytic) vibrio belonging to the El Tor group by Eisler &

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[1] As quoted by Hetsch (1928), two Japanese observers, Fukuhara & Ota, noting that extracts of typical cholera stools gave specific precipitin reactions with cholera-immune sera, recommended the use of such tests for the purposes of practical laboratory diagnosis. However as stated by Hetsch, no advantage has been taken of this theoretically interesting proposal. Recently Rainsford (1952) asserted once more that a precipitin test could be devised which would indicate the presence of cholera O antigen in stools of suspected cases thus affording a rapid means for the presumptive diagnosis of the disease.

Kovacs (1926) showed that no relationship existed between the precipitinogen of this organism and its toxin, which was merely apt to become adsorbed to the flocculi produced by the action of the precipitating immune sera. A further important result of these studies was that Eisler & Kovacs were able to demonstrate the presence of two components of the precipitinogen, a thermolabile and coagulable one which was adsorbable to animal charcoal and a thermostable component resistant to boiling heat.

It is of importance to refer at the present juncture also to attempts to distinguish between cholera and cholera like vibrios by precipitation tests with concentrated salt solutions. Liefmann (1913) following up casual observations made in this respect by Porges (1906) with ammonium sulfate preferred for his work magnesium sulfate used not only in varying concentrations for tube tests but also for slide tests. For the latter purpose, loopfuls of the cultures to be examined were thoroughly mixed with drops of concentrated magnesium sulfate solution and also—to guard against wrong positives through spontaneous agglutination of the vibrios—with drops of normal saline placed on slides results being read immediately

Liefmann obtained in this manner precipitations in the case of 12 out of 14 cholera strains, whereas out of 9 cholera-like strains 8 gave entirely negative results. In tube tests 30 out of 40 cholera strains were well precipitated with 90 / magnesium sulfate, 6 gave weaker and 4 negative results. Out of 20 cholera-like strains, only one reacted strongly while the strain reacting positively in the slide tests produced a trace of precipitation the other 18 strains gave negative results.

Greig (1913b) repeating such tests with a larger material, found that out of 176 cholera strains 164 were completely salted out, 12 only in traces, whereas out of 41 vibrio strains not agglutinable with cholera immune serum only 6 showed a strongly positive reaction, 8 reacted weakly and 27 were not at all affected. Thus, as concluded by Greig, a close but not an absolute parallelism existed between these and agglutination tests. It follows that the method of salt precipitation does not furnish fully reliable results.

Complement fixation tests

As can be gathered from the summaries of Köhlisch (1910) Kolle & Schürmann (1912) and Hetsch (1912) the early application of complement fixation tests for the purposes of cholera laboratory diagnosis (1906-07) soon led to considerable debates Apart from the question whether with the aid of this method a differentiation could be made between cholera and cholera like vibrios, it became at once a hotly contested point whether cholera and El Tor vibrios reacted identically or differently when tested in this manner with cholera immune sera.

Advocating the latter opinion, Markl (1906) maintained that he had observed complete complement fixation when testing classical cholera vibrios with the aid of a cholera immune serum, but only partial fixation

in analogous tests with El Tor strains—a difference which he ascribed to differences in the receptor apparatus of these two categories of strains.

Ruffer and Crendiropoulo (see Ruffer 1907) obtaining negative results in complement fixation tests with El Tor vibrios, which reacted like the classical cholera strains in agglutination tests with cholera immune serum, felt entitled to deny the specificity of the latter method

There can be no doubt however that the technique used by these workers for their complement fixation tests was unsatisfactory particularly because they used suspensions of living organisms as antigens As shown by Neufeld & Haendel (1907) it was impossible in this manner to obtain reliable results with haemolytic vibrios, because as a rule haemolysis became manifest after one hour in all tubes into which culture material of such organisms had been embodied If on the contrary El Tor suspensions killed by half an hour's exposure to 70°C were used, results comparable to those with non-haemolytic strains could be obtained [1] Neufeld & Haendel established in this manner that "the El Tor vibrios also in complement fixation tests with specific cholera sera react like true cholera bacilli"

Though a few subsequent workers again advocated views similar to those of Markl and of Ruffer, the validity of the findings of Neufeld & Haendel which were soon confirmed by Besche & Kon (1909) is now generally accepted. Indeed, in view of the fact that no or at least no marked differences exist in the antigenic structure of cholera and El Tor vibrios respectively it is impossible to assume that these two categories of organisms react differently in properly performed complement fixation tests

In addition to the above-described controversy some of the early workers, for instance Schütze (1907) Neufeld & Haendel (1908) Baerthlein (1912) Michiels (1913) and Pottevin (1913a) expressed doubts as to the usefulness of complement fixation tests for a differentiation of cholera and cholera like strains.

However the full specificity of the complement fixation tests and the parallelism of their results with those obtainable with the aid of the agglutina tion method has been asserted by numerous other cholera workers, e g. by Ballner & Reibmayr (1907) Bocchia (1911) Feldmann (1917) Kabeshima (1918a) and Mackie (1922) while Koshland & Burrows (1950) even came to the conclusion that the agglutinating and complement fixing antibodies to the vibrio O antigen were closely similar if not identical

It has to be admitted that the practical value of complement fixation tests in cholera-diagnostic work is limited, but this is due merely to the tediousness of the method and to the special technical requirements involved (Bocchia 1911) Nevertheless hand in hand with Pfeiffer's reaction or in the case of avirulent strains, even in place of the latter method, complement

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[1] The use of cholera vibrios killed by moderate heat (60°C) as antigens in complement-fixation tests, was also recommended in 1907 by Weil. Some Japanese cholera workers, such as Uyeda (1924) and Fujimori (1928), laid stress upon the use of well-boiled antigens so as to abolish the inhibitory action of a supposed impedin

fixation tests might still prove of value in dealing with atypical cholera strains.

It is of interest to add that in place of live organisms or preferably of killed culture materials, some special antigens have been used for cholera diagnostic complement fixation tests. Thus Rondoni (1910) established that the nucleoproteid of *V cholerae* was fully satisfactory in this respect and that, *vice versa* subcutaneous injection of rabbits with this material produced sera containing complement fixing antibodies. Kutscher & Schaefer (1916) proved through tests with rabbit immune sera that cholera vaccines formed suitable antigens for complement fixation tests. Proposals have been made by a few workers, first apparently by Nedrigailoff (1909) to expedite cholera diagnostic work by directly using the patients faeces as antigens in complement fixation tests

Nedrigailoff (1909) put fluid cholera stools into tall cylindrical glasses and after sedimentation used the supernatant as antigen. Complement fixation tests performed instead with Berkefeld-candle filtrates of cholera stools gave almost completely negative results. Since the same held true if fresh broth cultures or suspensions of fresh agar cultures of *V cholerae* were passed through the candles, whereas the filtrates of old cholera cultures proved to be suitable antigens in complement fixation tests, Nedrigailoff postulated that —in contrast to the latter materials—cholera faeces contained no endotoxins.

Tokunaga (1911), carrying out complement-fixation tests with the faeces of cholera patients and cholera-immune sera, obtained positive results with 79 / of his specimens. Faeces of cholera carriers gave, on the contrary invariably negative results.

It was claimed by Amako & Kojima (1912) that, if the supernatant of typical cholera stools was used as antigen in complement-fixation tests, a diagnosis could be arrived at in 7-8 hours. Atypical stools containing only few vibrios had little or no antigenic value and it was necessary in such cases to use the upper layer of 6- to 10-hour-old peptone water cultures made from these stools in order to obtain good results in complement fixation tests.

In view of the technical difficulties involved it is not surprising to find that no large-scale advantage has been taken of the above-described method of cholera stool examination even in the past. At present, when highly specific media are available for a rapid direct isolation of *V cholerae* from the stools, it is no longer possible to ascribe any practical value to it.

As stated by Svenson, some preliminary investigations by a Russian worker, Tuschinsky (1909) gave reason to hope that diagnostic advantage might be taken of complement fixation tests with known cholera antigens and sera of patients with signs of choleraic disease Amako & Kojima (1912) who further studied this possibility used as antigens the combined washings of 3 or 4 agar grown cholera strains. Making complement fixation tests with human sera, they obtained positive results in 15 out of 34 mild cholera cases, in the case of 20 out of 28 patients with moderate to severe attacks of the disease and in 5 out of 17 cholera carriers. Complement fixation tests with the sera of 2 patients with fulminant cholera and with those of 3 individuals with signs of cholera typhoid gave negative results. Commenting on these findings, Amako & Kojima stressed the necessity of using polyvalent

antigens of known activity, because—as has been generally acknowledged—different cholera strains may give variously marked results in complement fixation tests.

In view of this evidence it is undeniable that such tests might be used for establishing the diagnosis of cholera currently or perhaps rather retrospectively the more so because Yoshino (1922) established that complement fixation tests with known cholera antigens and normal human sera (as well as with normal rabbit or horse sera) invariably gave negative results Still, for practical reasons it appears more expedient to use agglutination tests, possibly also *in vitro* bactericidal tests in preference to complement fixation tests for this purpose

Balteano & Lupu (1914) found that complement fixing antibodies appeared in persons who had been vaccinated once against cholera 14 days after the injection, and after 9 days in those who had in the meanwhile received a second vaccine dose. In the former group the complement fixing property of the serum reached its maximum five days after it had become manifest and disappeared within two months as against three months in the case of the twice or thrice vaccinated.

Schoebl & Andaya (1925) who also performed complement fixation tests with the sera of cholera vaccinated persons, used for this purpose rather small vaccine doses, 28 of the individuals in question receiving one dose of 500 million 5 a single dose of 1000 million and 7 two doses of 500 and 1000 million respectively Nevertheless, as revealed by tests made one week or 12 days after vaccination, complement fixing antibodies became demonstrable practically always, the only exceptions being two individuals who had received a single dose of 500 million and were tested one week later As shown by repeated tests, the complement fixing antibodies were apt to persist for 6-10 months. Both the length of persistence and the titres reached seemed to depend to a higher degree upon the number of the injections given than upon the amounts of vaccine administered.

Phagocytosis tests

Though profound studies by Neufeld & Hüne (1906 1907) adduced evidence that specific bacteriotropic substances or as they are usually called, opsonins rendering the vibrios liable to phagocytosis were present in cholera immune sera, and further investigations by Neufeld & Haendel (1907) showed that El Tor vibrios were similarly influenced by the cholera tropins, few attempts have been made to utilize methods based upon these observations for the purposes of practical cholera diagnosis

Schütze (1909) who seems to have been the first to devote attention to such tests, evolved a technique of his own, described as follows

An exudate rich in leucocytes was produced by injecting guinea pigs intraperitoneally with 10 ml of broth in which 2 g of aleuronat had been suspended, and puncturing the peritoneal cavity of the animals 8 hours later The exudate thus obtained was suspended

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cholera vaccinated individuals Marked reactions (phagocytosis by 80% 100% of the cells) were noted in about two-thirds of this group

Allergic and skin tests

Shwartzman (1928) observed that if a preparatory intradermal injection of rabbits with a filtrate of *S typhosa* was followed 20-48 hours later by an intravenous administration of the same or a suitable heterologous bacterial filtrate in the majority of the animals tested marked haemorrhagic lesions developed at the site of the preliminary injection which were apt to undergo necrosis and ulceration

As first established by Gratia & Linz (1931) and confirmed by Uyeda (1934) and Vassiliadis (1935c), this curious, though not, or at least not strictly specific reaction known under the name of the Shwartzman phenomenon could also be produced by culture filtrates of *V cholerae*

Gratia & Linz (1931) found that this phenomenon could be produced not only in rabbits but also in guinea pigs through a preliminary intradermal injection followed by a second injection into the jugular vein or into the heart. They also drew attention to the similarity of Shwartzman's phenomenon with a reaction described by Sanarelli (1924a) according to whose observations a preparatory intravenous injection of rabbits with living cholera vibrios, followed 24 hours later by a second intravenous administration of either the homologous or a heterologous culture filtrate produced haemorrhagic reactions in the intestines, occasionally massive intraperitoneal haemorrhage, congestion of the genital organs apt to lead to abortion in pregnant animals, and sometimes immediate death.

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Vassiliades (1935c) exploring whether Shwartzman's phenomenon might be elicited with El Tor as well as with cholera vibrios, recorded the following results

	Number of animal	*Dose for preparatory injection*	*Dose for intravenous injection*	*Results positive*	*Results negative*
Filtrate of *V cholerae*	3 rabbits	0.25 ml	1 ml per kg body weight	2	1
	4 guinea-pigs	0.11 ml	0.75-1.25 ml for 400-600 g	0	4
Filtrates of *V El Tor*	6 rabbits	0.25 ml	"	0	6
	8 guinea-pigs	0 10 ml		0	8

It will be noted that (*a*) administration of *V cholerae* filtrates produced Shwartzman's phenomenon in rabbits, but—in contrast to the findings of Gratia & Linz—not in guinea pigs and (*b*) on the contrary filtrates of

in normal saline in centrifuge tubes and twice washed in such saline with the aid of centrifugation, the resulting sediment, suspended in 4-5 ml of normal saline, being used for the tests.

To perform these, 1 ml of the leucocytic suspension was mixed in centrifuge tubes with equal amounts of (*a*) cholera immune serum inactivated by heating for 20 minutes at 54°C, and (*b*) broth cultures of the vibrios to be tested. The mixtures were kept at 37°C for 10 minutes, then centrifuged for $\frac{1}{2}$ hour After the sediment had been twice washed in normal saline it was used for the preparation of smears which, after heat fixation, were stained for 3-5 minutes with alkaline methylene blue solution and then examined under the microscope in order to assess the degree of phagocytosis.

Carrying out such tests with a cholera and an El Tor strain as well as with three strains of cholera like vibrios (Metchnikoff I and II, and Finkler Prior) Schütze established that the former two vibrios were phagocytosed under the influence of cholera or El Tor immune sera to a considerably higher degree than the cholera like vibrios which were not at all ingested by the leucocytes when 1/50 dilutions of cholera immune serum were used. Analogously if a serum raised against *V metchnikovi I* was used, almost no phagocytosis of the heterologous strains resulted. However no such differences were apparent, if instead a serum raised against *V metchnikovi II* was used in a dilution of 1/20 Schütze postulated, therefore, that the specificity of the vibrio opsonins was not absolute and that, though he had been able to distinguish with the aid of his method between cholera and cholera like vibrios, this procedure alone should not be used for the purposes of differential diagnosis, agglutination tests, supplemented by Pfeiffer's reaction, remaining the " main criteria " for this purpose

Amako (1909) studying the opsonic properties of the sera obtained from 58 cholera patients or convalescents, came to the following conclusions

" 1) According to my tests with cholera vibrios normal sera and particularly cholera convalescent sera showed marked opsonic effects.

" 2) If fresh undiluted convalescent serum was used, the cholera vibrios were lysed extracellularly so that opsonic effects were not noticeable if however serum dilutions were used, one could observe a clear [*deutliche*] opsonic action

" 3) If the bacteriolytic property of the serum is too strong, so that one cannot recognize an opsonic action even if serum dilutions are used, one can observe an action after inactivation of the serum (through heating for 15 minutes at 60°C), because the cholera opsonins, like other immune-opsonins, are thermostable " [Trans.]

As shown by Amako with the aid of numerous graphs, there existed in individual cases, as a rule a parallelism between the agglutinatory, bacteriolytic, and opsonic properties of the sera.

It is of interest and of some practical importance to add that according to the investigations of Eisele McCullough & Beal (1948) already referred to above opsonophagocytic tests with brucellae made according to the method recommended in the handbook on brucellosis in animals and in man of Huddleson (1943) were found to give positive results in 16 out of 20

cholera vaccinated individuals Marked reactions (phagocytosis by 80% 100% of the cells) were noted in about two-thirds of this group

Allergic and skin tests

Shwartzman (1928) observed that if a preparatory intradermal injection of rabbits with a filtrate of *S typhosa* was followed 20-48 hours later by an intravenous administration of the same or a suitable heterologous bacterial filtrate in the majority of the animals tested marked haemorrhagic lesions developed at the site of the preliminary injection which were apt to undergo necrosis and ulceration.

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two different El Tor strains failed to elicit this phenomenon It would be of interest to establish with the aid of a larger material whether this diffe rence between cholera and El Tor vibrios holds generally true

Continuing investigations in this field, Raynal Lieon & Fiessolle (1940) were able to produce local skin reactions in guinea pigs, which had been immunized one month previously through intraperitoneal injection of live cholera vibrios through intradermal administration of cholera antigens obtained with the aid of trichloracetic acid extraction. Normal guinea pigs, receiving such antigens by the intradermal route failed to react.

Kovacs (1932) reported that intracutaneous administration of the toxin of El Tor vibrios produced marked local reactions, consisting of infiltrations and hyperaemia or, if higher doses were used, even of necrosis, in the skin of rabbits, guinea pigs and, as he showed on himself also in the human skin. The appearance of these reactions could be inhibited by the simul taneous administration of sufficiently large doses of antitoxic sera and the reactions remained absent in guinea pigs which had been immunized with the toxoid of the *V kadiköj*, unless toxin amounts ten times exceeding the *dosis necroticans* were used

Kovacs maintained that such intracutaneous tests could be used for a differentiation of El Tor and related vibrios from *V cholerae* because administration of 0 1 ml of the centrifugate of 6-day-old cholera broth cultures produced no skin reactions in a guinea pig whereas such reactions could be elicited with the centrifugates of El Tor and Kadiköj vibrios.

In contrast to these findings, Yu, Chen & Chen (1932) obtained positive skin reactions with toxic solutions prepared by (*a*) growing typical non-haemolytic cholera vibrios, the virulence of which had been enhanced by repeated guinea pig passages, in buffered glucose-free broth (*b*) centrifuging the culture fluid and filtering the supernatant through N Berkefeld candles. Reporting on their findings, Yu, Chen & Chen stated that

(*a*) three normal rabbits intradermally injected with 0 1 ml of such a filtrate, showed skin reactions of considerable size

(*b*) tests on 6 rabbits which had been immunized either with the toxic filtrate or with cholera vaccine, gave negative results

(*c*) tests on human volunteers, who received 0.1 ml of the toxic filtrate intradermally into one forearm and, as a control, 0.1 ml of such a filtrate heated for 2 hours at 100°C, gave the following results

	Positive	*Negative*	*Total*
Vaccinated	1	27	28
Not vaccinated	60	3	63
Total	61	30	91

* Positive reactions appeared within 6-12 hours, reaching a maximum between 20 and 24 hours and fading in 48 hours.

Yu, Chen & Chen suggested that their method might prove of value in testing individual susceptibility to cholera and also for checking the length

of the immunity produced by cholera vaccination They noted in this connexion that the single vaccinated individual who gave a positive skin test had received cholera vaccine about three years before he had been examined.

The usefulness of skin tests for assessing the value of cholera vaccinations was also upheld by Brounst & Maroun (1949) who obtained positive reactions in 5 out of 10 thrice vaccinated individuals tested by intradermal injection of 0 1 ml doses of the vaccine Appearing after 48 hours, these reactions consisted of local congestion and oedema sometimes accompanied by the appearance of a central necrotic zone or by a nodular infiltration

Large-scale use of skin tests was made by Sabry (1950) during and after the 1947 cholera outbreak in Egypt. The antigen for these tests was prepared by (*a*) simultaneously growing three cholera strains in broth (*b*) killing the organisms by exposure to 52°C for one hour and (*c*) centrifugation the lower more concentrated part of the centrifugate being used in a dilution of 1/300 Frankly positive results obtained with this antigen through intradermal injection consisted in the appearance of an oedematous papule surrounded by an erythematous zone, the reaction becoming fully developed after 24 hours and then disappearing within 48 hours. In the case of mild reactions, no or only an ill-defined erythema became manifest round the papules.

Tests made in a cholera hospital on 13 patients, 9 convalescents, and 7 carriers produced only mild reactions a result ascribed by Sabry to (*a*) a supposed state of allergy in these individuals, and (*b*) massive doses of sulfaguanidine which they had received. Sabry stated in the latter connexion that in the course of his further work he had succeeded in rendering the skin test negative by administering to a group of originally positive individuals 6 g of sulfaguanidine daily for 9 days.

Further results recorded by Sabry may be tabulated thus

Groups tested	*Reactions* positive	mild	negative
5 stool-positive cholera carriers	5	—	—
37 vibrio-positive carriers	20	—	17
265 non-cholera patients	9	20	236
186 members of the hospital staff	32	51	103

Note 31 individuals giving originally a negative skin test, remained negative when re tested 7 30 days after they had received one dose of cholera vaccine.

Commenting upon further observations, Sabry stated that

> the percentage of the positive cases in the more recent experiments decreased among the domestic hospital staff as well as among ordinary cases, indicating that at least some of the carriers are on their way to recovery As a decisive proof of the validity of this observation, 1001 cases were inoculated (i.e. skin-tested) during the period from 27-4-1948 to 6-7 1948 without the occurrence of one single positive reaction."

Sabry emphasized, therefore the significance of the skin tests, maintaining that " the persistently positive cases represent the dangerous carriers responsible for the propagation of epidemics, who should be detected and kept under strict control " However apart from the fact that most workers do not share Sabry s belief in a dangerous role played by carriers in the spread of cholera it has to be kept in mind that in a majority of his observations he seems to have been unable to correlate positive skin tests with findings of *V cholerae* in the stools of the individuals concerned.

Natural Immunity

Resistance and natural immunity

As shown by ample experiences, and well illustrated by the classical observation of Macnamara (1876) that out of 19 persons drinking water from a vessel which had been accidentally polluted with fresh cholera excreta, only five actually contracted the infection, the ingestion of materials containing *V cholerae* is by no means invariably followed by clinical manifestations of the infection General agreement exists, however that such a non-appearance of the disease is due if not solely at any rate mainly to an unspecific resistance to the infection instead of being the result of a specific natural immunity

Various factors contribute to the unspecific resistance against cholera infection. As has been noted in the preceding chapter some evidence has been adduced to show that the saliva of healthy persons exerts an antibacterial action on *V cholerae* and might thus form a first line of defence against not too massive infection. Be this as it may it is certain that the acidity normally prevailing in the stomach forms a potent barrier against the entry of cholera vibrios into the intestines where owing to the presence of an alkaline reaction conditions are favourable to the multiplication of the organisms. There can be no doubt, however that even in the intestines unspecific defence mechanisms against cholera infection exist. The competition of the normally present bacterial flora is apt to exert an influence in this respect. Moreover it is likely that a normal condition of the intestinal mucosa is capable of preventing an entrenchment of the invaders Whether this defence mechanism is vested in the normally present mucous coating of the mucosa, as maintained, for instance by Harvey (1929) or dependent upon the intactness of the epithelium itself (Romano 1912) is difficult to decide Probably both these factors play a role to a varying extent.

There can be no doubt, however that the protection afforded to individuals in full health through the above-described means of an unspecific resistance to cholera is relative in degree Kolle & Schürmann (1912) discussing this problem, pointed out with much reason that (*a*) like other acidophobe bacteria, cholera vibrios enclosed in copious amounts of food are apt to escape the action of the acid gastric juice and (*b*) infected fluids,

particularly cold drinks are apt rapidly to pass the stomach particularly the empty stomach Experimental observations supporting the latter contention have been noted already in Chapter 3 Kolle & Schürmann insisted also that even in normal persons the acidity of the gastric juice was subject to variation and could be low at times.

While thus even healthy persons are by no means invariably proof against an entry of *V cholerae* into their system, ample observations have shown that individuals with a permanently low acidity of their gastric juice are particularly apt to fall victim to cholera infection Thus it is a well established fact that the disease is particularly rampant among individuals suffering from chronic gastritis due to the habitual abuse of alcoholic drinks. Sticker (1912) quoted in this connexion the observations made by Adams (1849) during an 1848–49 outbreak at Glasgow, according to which cholera killed 91 out of 100 drunkards as against 19 out of 100 abstemious persons. That temporary gastro-intestinal disturbances also favour cholera infection has been confirmed by observations on a greatly increased frequency of cholera admissions on the days immediately following Sundays or holidays, recorded during various outbreaks. Sticker drawing attention to these records also noted that according to several of the early observers the use of emetics and also that of even small doses of laxatives seemed to promote cholera infection.

The concept suggested by these observations that, besides the normally prevailing acidity of the gastric juice a normally present unspecific resist ance of the mucous surface of the intestines prevents cholera infection, is supported by laboratory experiences. As will be fully discussed in the sixth chapter several workers have succeeded in producing syndromes similar to perhaps even identical with, human cholera in experimental animals ordinarily not amenable to oral infection with *V cholerae*—in part by creating conditions analogous to those found to promote the appearance of cholera in man. Thus Pottevin & Violle (1913) recorded success in this direction by administering saline purgatives to monkeys before oral cholera infection and Cantacuzène & Marie (1914) noted the appearance of a syndrome corresponding to that of human cholera in guinea pigs the resistance of whose intestine had been lowered through administration of podophyllin. Two guinea pigs which had been dosed with this drug contracted the disease by mere contact with cholera infected animals.

Most authorities are sceptical, and many frankly deny that in addition to the well-documented presence of unspecific defence mechanisms a specific natural immunity against cholera infection exists As far as it is permissible to adduce in this respect the evidence supplied by tests with the sera of normal non vaccinated subjects, it speaks against the presence of such a specific immunity immune bodies being either altogether absent or demonstrable only at negligibly low titres

Ample observations made in parenterally infected animals, though not directly applicable to the problem presently under review, are of interest in so far as they showed up a fundamental difference between an unspecific resistance and a specific immunity to cholera infection It was found that previous administration of heterologous bacteria such as *Chromobacterium prodigiosum*, *Proteus* and *Ps pyocyanea* could protect guinea pigs against intraperitoneal injection of lethal doses of *V cholerae* However as shown by classical investigations of Pfeiffer & Issaeff (1894) the protection afforded in this manner was distinct from the specific immunity produced by the *V cholerae* by its early appearance and rapid disappearance as well as by the failure of the non-specific organisms to produce cholera immune sera.

Naturally acquired immunity

While it was maintained by some early observers that persons who had survived a cholera attack had become permanently immune against this infection, Koch (1884) held that this acquired immunity

" does not seem to persist for a long time because there is a sufficiency of examples to show that an individual who had been affected during one epidemic fell ill with cholera a second time during another outbreak but one hears but rarely that somebody had been attacked twice during the same cholera epidemic " [Trans.]

However Sticker (1912) emphasizing that several workers had observed cholera attacks in individuals who had recovered from the disease but some weeks previously (*Spätrecidive*) denied the development of a specific immunity against this infection and postulated that the rarity of second attacks was due merely to extrinsic causes, particularly the infrequency and short duration of the epidemics. Harvey (1929) discussing this problem, also adopted a cautious attitude, stating that

" the probabilities against an individual being in a position to contract a second attack of cholera must be great. This does not apply merely to his being in contact with cases of cholera, but to the likelihood of any individual s contracting infection even after the ingestion of the cholera vibrio "

Nevertheless, Harvey felt convinced that cholera attacks produce a naturally acquired immunity but qualified this statement by adding that " but little information exists as to the longer or shorter duration of that immunity "

As far as one can judge from the scanty evidence available on this point, it appears that cholera attacks, though not rendering the individuals concerned permanently immune protect them against the infection for several years. This view was advocated for instance by Salimbeni (1915), who stated that

" the cured cholera patients are without any doubt immune to cholera for a longer or shorter time, because, though one knows of comparatively quite rare instances of individuals who had the disease two or even three times some years apart, as far as I know no cases have been recorded of persons attacked by well-characterized cholera during one and the same epidemic " [Trans.]

Similarly it was recently stated by Maxcy (1951) that

"an attack of cholera does not necessarily confer protection against a subsequent attack. Nevertheless, second attacks within a period of a few years are uncommon."

A most interesting and important question arising in this connexion is whether, as considered probable by Harvey, the inhabitants of cholera endemic areas by suffering from slight and unnoticed attacks, become immune to the infection It would be highly desirable to study this problem through large-scale investigations made in truly endemic areas with the aid of the immunological and experimental methods now available

Dealing with the information then available on the presence of immune bodies in the sera of cholera patients and convalescents, particularly the experiences of Svenson (1909) referred to above, Hetsch made in 1912 the following statement

"The experience that the agglutinin and bacteriolysin content of the blood in man and animals is considerably higher after cholera vaccination than after spontaneous cholera attacks and that nevertheless even after the slightest spontaneous attack the immunity is very considerably higher than after vaccination justifies the assumption that recovery from cholera produces a local immunity of the intestine, which is not produced to such a degree in animal experiments and through vaccination." [Trans.]

Plausible though this assumption is, it appears that Hetsch, again dealing with the problems of cholera immunology in 1928 laid no more stress upon a local immunity as contrasted to a systemic immunity against the infection. Be this as it may it is certain that persons who have recovered from cholera can remain immune to the infection, even though no immune bodies are demonstrable in their sera.

Active Induced Immunity

Introductory remarks

The first attempt to confer protection against cholera through a method of active immunization was made by Ferrán (1885) during the epidemic rampant in Spain during 1884 Noting that guinea pigs which had survived an injection of living cholera vibrios cultivated from faeces in broth were resistant to administration of further doses lethal to untreated animals he applied this method to man. Ferrán made for this purpose initial injections of 8 drops of a broth culture of *V cholerae* to which bile had been added, and administered at intervals of 6-8 days two further doses of 0.5 ml each.

As summarized by Kolle (1896b) and by Voges (1896), it was soon shown by several workers that Ferrán worked with impure cultures containing only a minority of cholera vibrios besides numerous contaminating organisms It is not surprising, therefore that his method of vaccination not only gave no satisfactory results, but often produced severe, according

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the use of virulent strains being advantageous at least in so far as these are bound to be smooth and therefore antigenically suitable However one must fully agree with Kolle s contention that the use of live instead of killed cholera vaccines offers no advantage in so far as, according to Haffkine s own observations the organisms contained in his *virus fixe exalté* succumbed soon after injection The action of live vaccines depended therefore not upon a survival of the organisms but upon the liberation of immunologically active substances contained in their bodies, as Kolle believed, of the toxins

Far more important than these considerations were the results of comparative determinations of the bactericidal properties of the sera obtained from persons who had been vaccinated by Kolle (1896a 1897) either three times according to Haffkine s method or once only with killed cholera vaccines, i.e , with suspensions of cholera cultures exposed (*a*) to heating at 56 C for one hour, or (*b*) to the action of chloroform vapours Kolle established in this manner that the bactericidal titres of the sera derived from the individuals once injected with killed cholera vibrios were as high as or even higher than those of the persons thrice injected with Haffkine vaccine. This held true of the determinations made 10 days after the completion of vaccination as well as of those which could be made one year or even longer afterwards. Kolle (1896a) summarized, therefore, that

" since an equal effect is obtained, it is better to use *sterile* cholera vaccines than vaccines containing *living* vibrios, because the manufacture of the latter (production of the cultures) is difficult as well as dangerous. Further the pain produced by the injections deters many from submitting to a second inoculation. We know through my investigation that *one* injection of a somewhat larger dose produces the same effect as multiple inoculations." [Trans.]

It is somewhat surprising to find that in spite of these observations of Kolle a few subsequent observers again advocated the parenteral use of live cholera vaccines. Thus Nicolle Conor & Conseil (1912) used living virulent cholera vibrios for intravenous [1] injections

As summarized by Hetsch (1928) these workers prepared their vaccines by subjecting suspensions of 20-hour-old agar cultures to centrifugation followed by repeated washing and final resuspension of the sediment, one drop of the fluid thus obtained being adjusted to contain 4 million of *V cholerae* This amount, diluted in 50 ml of normal saline was used as the initial dose for adults and this was followed 10-15 days later by administration of a dose six times stronger

Nicolle and co-authors stated that the 36 persons thus vaccinated showed no serious reactions, particularly no diarrhoea. The application of the vaccine led to the abundant formation of antibodies in the sera of these

[1] The method of *intravenous* administration of cholera or mixed vaccines was also recommended by Quarelli (1917). He found that this method of vaccination, while causing but mild reactions, led to a more rapid and persistent production of antibodies than the subcutaneous method.

to some observers occasionally even fatal, reactions. Modern writers are nevertheless unanimous in stating that Ferrán, though acting rather injudiciously, deserves credit for having first demonstrated the possibility of an actively induced immunity against cholera

It was the great merit of Camaleia (1888) to have first shown that it was possible to protect guinea pigs against lethal doses of *V cholerae* not only with the aid of living cultures the virulence of which had been reduced, but also with growths *killed* by heating at 120 C. Curiously enough, the great practical importance of the latter observation was at first overlooked, Haffkine (1892b) recommending once more a method of cholera vaccination based upon the use of living organisms.

Following the scheme successfully used by Pasteur for rabies prevention Haffkine used two cholera vaccines of different strength, administering first a " weak virus " obtained through cultivation of cholera vibrios under continuous aeration at 39 C, and five days later a *virus fixe* consisting of organisms the virulence of which had been exalted through repeated intraperitoneal passage directly from guinea pig to guinea pig. As summarized by Hetsch (1912) Haffkine used originally a suspension of a tenth part of a slant, prepared with boiled water as a dose for adults, and gave to children 1/20th and to infants 1/100th part of a slant, but afterwards repeatedly changed these dosages, using for instance 1/12th and 1/8th of slants of the weak and the exalted virus respectively (Voges 1896) Later on in order to reduce the reactions and so to win the goodwill of the population, Haffkine often used lesser doses (1/20th of a slant)

The difficulties of using Haffkine s method of cholera vaccination on a large scale were tremendous. As he admitted himself (see Kolle, 1896a) it was a particularly heavy task to keep sufficient amounts of *virus fixe* available through continuous animal passages. In large-scale practice it also proved often impossible to administer second doses, so that only one third of the 40 000 persons inoculated up to 1895 in India according to Haffkine's method received these Nevertheless some of the records published by him leave no room for doubt that his method was apt " to protect man against natural cholera infection " (Kolle, 1896b)

However while admitting the value of Haffkine s method, Kolle (1896b) maintained that not only difficulties met with in manufacture but also considerations of a principal nature spoke against the use of live cholera vaccines. Believing that the toxins of *V cholerae* were instrumental in conferring immunity he considered it unnecessary to use strains of a particularly high virulence for vaccination, because according to the observations of Dungern (1895) virulent cholera cultures were no more toxic than avirulent ones. It has to be noted in this connexion that, since according to the now-accepted views it is the antigenic structure and not the toxicity of the strains used for vaccine manufacture which is of decisive importance, the above contention of Kolle is no longer fully acceptable

Though the phenomena of dissociation were still unknown at the time it can be gathered from the summary of Hetsch (1928) that most workers engaged in the manufacture of cholera vaccines during the First World War insisted upon the use of freshly isolated strains It was usually recommended to select several of these for the preparation of polyvalent vaccines.

Schwarz (1919) one of the workers quoted by Hetsch, aptly distinguished between vaccines manufactured from strains isolated locally during a cholera outbreak (*epidemieeigene Impfstoffe*) and those prepared from strains of a heterologous origin (*epidemiefremde Impfstoffe*)—either polyvalent vaccines or vaccines made from single selected strains. The criteria for choosing such specially suitable strains established by tests with individually prepared vaccines, were according to Babes (1914) (*a*) absence of a severe reaction after vaccination and (*b*) marked immunizing properties, as shown through tests in guinea pigs injected simultaneously with dilutions of the sera of persons who had been given doses of the vaccines in question, and lethal doses of *V cholerae*

In the experience of Schwarz it was of importance to use vaccines prepared locally during the epidemic to be dealt with He noted in this connexion that individuals who contracted infection even though they had been injected with such vaccines had slight attacks or merely became carriers of *V cholerae*, whereas usually severe forms of the disease were observed in persons who fell ill with cholera though they had received injections of heterologous vaccines

The necessity of using fully smooth cholera strains for vaccine manufacture was stressed by Steward (1933) He noted that in actual practice during the off seasons eight subcultures were made from a recently isolated strain which were kept in the refrigerator[1] and successively used to prepare vaccines. During the epidemic seasons freshly isolated smooth strains were used for this purpose and were frequently replaced by strains of the same character Thus, as Stewart stressed, the old method of preparing cholera vaccines from stock strains had been given up Many of the subcultures made from the latter showed roughness.

Dealing again with the problem of selecting cholera strains for vaccine manufacture Taylor Ahuja & Singh (1936) stated that

"The maximum degree of protection in animals against infection with strains of the prevailing serological type is obtained by the use of vaccines prepared from strains which show both H and O agglutination with a serum of the Japanese original type and which also show the chemical structure (Linton s groups I and II) characteristic of the majority of agglutinable vibrio strains isolated from cases of cholera in India. Agglutinable strains from carriers and agglutinable variants produced from strains of origin other than cholera cases give a lower degree of protection."

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[1] The statement made in the text of Stewart s article that these subcultures were kept in the incubator is obviously due to an error in translation, because he noted that, in contrast to cholera cultures kept in the refrigerator those left t room temperature or in the incubator had a great tendency to become rough.

individuals and three of them remained healthy when afterwards given virulent cholera vibrios *per os*

Castellani (1913) stated that he had prepared a live attenuated cholera vaccine for subcutaneous injection by heating 48-hour-old cultures for one hour at 48 C or 45°C. However these vaccines produced much more severe local and general reactions than killed cholera vaccine and a further disadvantage was that the attenuated vaccines had to be used soon after preparation, because even if heating at only 45°C had been resorted to the organisms died within two months. One may truly say, therefore that there is nothing to recommend the use of Castellani s live vaccine in actual practice The method of Nicolle and colleagues seems to be capable of producing a solid immunity but its large-scale use would be fraught with great difficulties and even some danger It is not surprising therefore that —apart from some attempts to practice oral vaccination against cholera— parenteral administration of killed vaccines or to a lesser extent, of extracts prepared from *V cholerae* in various ways, has been adopted as the standard practice for large-scale vaccination campaigns. The various methods used for this purpose, and also the problem of oral vaccination, will now be dealt with seriatim.

Agar grown killed vaccines

Agar-grown killed vaccines have been continuously used for the pur poses of cholera control since their recommendation by Kolle in 1896 first on a large scale during the 1902 cholera epidemic in Japan (Murata 1904 Takano Ohtsubo & Inouye 1926)

While it would be redundant to enter into a detailed description of the technique of preparing agar grown vaccines, which is set forth in the textbooks on bacteriology and laboratory methods, it is of importance to discuss the following special problems of their manufacture standardization administration, and storage.

(1) *Choice of strains* Dealing with the problem of selecting strains suitable for the manufacture of cholera vaccines, Hetsch (1912) noted that some of the early workers had insisted upon the necessity of choosing highly virulent strains, while others reached a contrary opinion, pointing out that part of the avirulent strains had equally good antigenic properties Agree ing with the latter view Hetsch emphasized that the immunizing properties of cholera strains did not run parallel with their virulence and that it was essential, therefore to select those strains which showed a marked antibody formation in preliminary experiments. However as will be stated below some workers have recently again laid stress upon a high virulence of the strains used for the preparation of cholera vaccines As noted before it is certain that the use of freshly isolated (and, therefore presumably virulent) strains is of great importance in so far as these are unlikely to have under gone a loss in antigenic properties through roughening.

"Each of 50 mice was inoculated with the prescribed two doses of vaccine prepared from a single Inaba-type strain. A fortnight after the final dose half of this vaccinated group was challenged with 10 m.l.d. of the Inaba-type strain, and half received 10 m.l.d. of an Ogawa-type strain A similar number of mice inoculated with 2 doses of Ogawa type vaccine were divided into two groups, which were challenged with 10 m.l.d. of Inaba and Ogawa-type vibrios respectively All vaccinated mice survived."

Though in the opinion of Ranta & Dolman these findings did not necessarily imply that the type specific O antigens played a part in mouse protection, they pointed nevertheless to the existence of a cross protection between the two subtypes of *V cholerae*

This postulation was fully supported by Burrows et al (1947) who concluded from large-scale active and passive immunization experiments that there existed "complete cross protection between vibrio types"

For a further study of this problem, Ahuja & Singh (1948) injected guinea pigs subcutaneously with two doses of cholera vaccines prepared respectively from strains of the two subtypes or with a mixed Inaba Ogawa vaccine and challenged the animals 10 days after the second injection intraperitoneally with mucinized suspensions of live cholera vibrios of either the Inaba or the Ogawa subtype Tabulated, the results of these tests were as follows

Vaccine used	*Number of guinea-pigs immunized*	*Type of challenge strain*	*Survivals up to 96 hours Number*	*(%)*
Inaba	15	Ogawa	14	93 0
Inaba	15	Inaba	14	93.0
Ogawa	14	Ogawa	14	100 0
Ogawa	15	Inaba	13	89 0
Inaba+Ogawa	15	Ogawa	15	100 0
Inaba+Ogawa	15	Inaba	15	100 0

Note None of the 30 non vaccinated controls challenged with either the Inaba or Ogawa type vibrios survived.

Though considering that the results obtained with the mixed vaccine were not significantly different from those obtained with the two monovalent vaccines Ahuja & Singh concluded that

"On the basis of these findings the use of both sub-types of *V cholerae* for the preparation of prophylactic cholera vaccine would be more satisfactory than the use of either an Inaba or an Ogawa sub-type alone"

Dealing not only with the problem presently under review but with the selection of strains for the purpose of cholera vaccine manufacture in general, Pandit (1948) made the following important statement

"In view of the evidence regarding the prevalence of subtypes of vibrios in India, the vaccines used in the country are prepared from both the types of vibrios, particularly those isolated from fatal cases of cholera. It is customary in most laboratories to replace the strains used by new ones as they are isolated. Pending further information on the question of virulence of vibrios, this procedure was considered to be the most suitable for adoption in the manufacture of cholera vaccines. However it would seem that with

Quoting recommendations made by the Cholera Advisory Committee of the Indian Research Fund Association, Taylor (1941) stated more specifically that

"The strains ordinarily used in manufacture of (cholera) vaccine should show the following characters

"(a) Typical smooth translucent colony appearance.

"(b) Stable in salt solution.

"(c) Serological characters of O group I (Gardner and Venkatraman) sub-type Inaba and should agglutinate to titre with a serum prepared against the dried O Inaba antigen issued from the Standards Laboratory Oxford.

"(d) Producing acid from mannose and saccharose but not from arabinose.

"(e) Non-haemolytic."

Taylor added that in the opinion of the Cholera Advisory Committee there was no evidence to show whether the use of multiple strains for cholera vaccine manufacture was necessary or not. However since in certain areas strains of the Ogawa subtype had been isolated from a considerable number of cholera cases during epidemics, it was in the opinion of the Committee "for consideration whether strains for the Ogawa subtype should be incorporated in the vaccine and this is the practice in the Madras Presidency"

Yu (1938 1942) laid emphasis upon selecting fully virulent strains for the manufacture of cholera vaccines.

He recorded in this connexion the following results obtained when (*a*) injecting groups of mice at intervals of 3 days with 2 doses of heat-killed cholera vaccines (1000 and 2000 million respectively) which had been prepared individually from 6 strains varying in virulence, and (*b*) challenging the animals 20 days afterwards with 3 MLD of one of these strains

Vaccine	*Virulence of organisms*	*Number of mice tested*	*Survived*
(a)	++++	40	40
(b)	++++	40	38
(c)	+++	40	31
(d)	+	47	18
(e)	–	48	18
(f)	–	20	8

Yu added that stock cholera cultures were not suitable for vaccine manufacture because they were not perfectly smooth. They could be rendered negative to Millon tests by repeated mouse passages but these led to only a slight rise of the virulence of the strains.

During the following years considerable attention was paid to the question whether a cross-protection existed between the Inaba and Ogawa subtypes of *V cholerae* and whether consequently monovalent cholera vaccines or vaccines consisting of a mixture of equal parts of vaccines prepared from Inaba and Ogawa strains respectively should be issued.

Ranta & Dolman (1944) recorded in this connexion the following observations

organisms were no longer viable whereas in broth the overwhelming major ity of the vibrios were still in the stage of multiplication at that time

As generally agreed, an incubation at 37°C for 20-24 hours is suitable for the mass production of *V cholerae* in the course of vaccine manu facture

(3) *Killing methods* The first, and until rather recently the most amply used, method for killing the vibrios in the course of cholera vaccine manu facture was to expose the organisms to heat. As noted above Gamaleia (1888) resorted in his pioneer work to a temperature of 120°C Fairbrother (1928) established that the substance of *V cholerae* which on inoculation gave rise to protection in animals was heat stable, withstanding an exposure to 100°C for one hour Uyeda (1922) claimed that a "koktoantigen," i e. the supernatant obtained through centrifugation of *V cholerae* suspensions which had been boiled for 15-30 minutes, was the best cholera vaccine.

Though it is not possible to share this view, it is noteworthy that accord ing to the observations of Taylor (1936) Burrows et al (1947) and Singer (1948b) prolonged boiling exerted no untoward influence on the immunogenic power of cholera antigens

Notwithstanding these observations, the general tendency has been to use for heat killing the organisms in actual cholera vaccine manufacture even lower temperatures than that of 56°C recommended by Kolle in 1896 Haffkine (1913) who in about 1911 began to use a killed cholera vaccine, resorted for its preparation to an exposure of the vibrio suspensions to only 50 C for a few minutes but followed this procedure by the addition of 0.5% phenol. Generally speaking, however temperatures of 53 C to 54°C have been applied for the period of one hour If carefully implemented, this method gives fully reliable results, particularly if, according to the generally adopted practice, phenol to a final concentration of 0 5% is added as soon as initial samples have been withdrawn to test the sterility of the brews.

As summarized by Hetsch (1928) and Harvey (1929) methods to effect sterilization of agar-grown vaccines through the addition of antiseptics instead of through the application of heat have been recommended by several workers, the substances used being—besides 0 5% phenol—chloroform, ether formol, glycerol, hydrochloric acid, and quinine Singer Wei & Hoa (1948b) found an alcohol killed vaccine as satisfactory as those killed by heat or other methods.

Tentative use of 0 5% phenol was made already early in his work by Haffkine with the idea of abating the virulence of the cholera strains used for the preparation of his "weak virus" However as noted by Hetsch (1912) it was soon shown by some other workers that this chemical exerted a sterilizing instead of an attenuating effect. Large scale advantage of the

the development of the technique for the measurement of antigenicity of vibrio strains, it should be possible to select such strains only for vaccine production as show a sufficient high degree of antigenic potency Recently Ranta & Dolman (1944) and subsequently Burrows and his collaborators (1947 p 157) obtained evidence to show that practically complete cross protection exists between the two sub-types of cholera vibrios. However recent observations by Venkatraman in the King Institute tend to show that this may not always be the case, particularly if minimal quantities of antigens are used for protection."

As will be discussed below, the conclusion tentatively reached by Venkatraman was vigorously supported by Sokhey & Habbu (1950b) who denied that a cross-protection existed between the Inaba and Ogawa subtypes.

Under these circumstances it seems indicated for the present to use both Inaba and Ogawa strains for the manufacture of cholera vaccines destined for wide distribution Though as shown by observations like those of Burrows et al (1947) and of Sokhey & Habbu (1950b) the virulence of cholera cultures may be preserved for prolonged periods through freeze-drying (lyophilization) it is certainly best to replace the strains used for vaccine preparation by recently isolated ones whenever possible.

(2) *Cultivation methods* While, generally speaking, the high-quality agar media available for diagnostic work are also utilized for the manufacture of cholera vaccines some workers recommended for the sake of economy cheaper media, for instance, one prepared with 3% yeast instead of with meat or meat extracts and peptone (Fischer Bitter & Wagner 1915) However Ungermann (1917) giving a systematic description of the methods of cholera and typhoid vaccine manufacture in the Berlin Gesundheitsamt, warned against the use of cheap substitutes, particularly prefabricated meat extracts which because possibly made from meat of doutful freshness, were apt to contain products of protein decomposition and thus to cause untoward reactions in the vaccinated. Since, moreover media made with fresh beef gave a more abundant growth than those prepared with horse meat, the former alone were used for vaccine manufacture in the Gesundheitsamt. However as shown by the excellent quality of the cholera vaccine made in the Kasauli Institute in India, in countries where the use of beef for media preparation is out of the question mutton digests are apt to prove equally advantageous

While, in general, Roux bottles or similar containers or as recommended by Ungermann, large covered glass dishes (diameter 21 cm) are used in vaccine manufacture, Fischer and collaborators (1915) advocated the use of tin dishes They admitted, however that these became rusty after they had been used several times

Ungermann claimed that more abundant yields were obtained when, instead of suspensions made directly from agar subcultures, *ad hoc* subcultures made from these in broth (*Bouillonvorkulturen*) were used for seeding the flasks or dishes He adduced as explanation that in agar cultures of *V cholerae* even after a growth for only 24 hours a part of the

by Wright in 1902 and haemacytometer counts recommended about the same time (see summary by Soltmann, 1915) As is generally known the principle of the former method is to mix equal volumes of the bacterial suspensions to be tested and of fresh human blood in a capillary tube, to prepare stained smears from this mixture and to compare the number of bacteria present with that of the blood corpuscles. Since it may be taken that 5 million of the latter are present per ml this standard value can be used easily to compute the number of organisms per millilitre

Though the present writer for one cannot agree with the assertion sometimes made that Wright s method gives inconsistent results, it is generally admitted that the number of organisms elicited with its aid is lower than that actually contained in the bacterial suspensions under test. There can be little doubt, therefore that haemacytometer counts, which give exact values, are preferable for vaccine standardization but unfortunately simple as the implementation of this method seems at first glance, it is fraught with considerable technical difficulties and thus reliable only in the hands of experienced workers. It is under these circumstances of great importance that, as shown by ample experiences, results approaching or even equalling in exactness those obtained by dry weight determinations or properly made counts may be obtained with the aid of opacity tests.

No doubt it would be simplest to carry out the latter by comparing the opacity of the bacterial suspensions under examination with that of previously prepared vaccines. However though used by some workers, in the experience of most observers this method is as unreliable as it is expedient, because bacterial vaccines, quite particularly cholera vaccines, are apt to undergo a process of autolysis, thus changing in aspect and density It is necessary therefore, to carry out the opacity tests with the aid of standard suspensions of a non bacterial nature preferably those of a stable character which can be used on successive occasions.

Various substances have been suggested or actually used for this purpose Ungermann (1917) noted in this respect that emulsions of lecithin in water though formerly recommended for opacity tests, possessed in higher concentrations a colour of their own which interfered with their suitability for the standardization of cholera vaccines. He recommended, therefore the use of alcohol dilutions of a 10 % solution of dry Canada balsam in benzol. A suspension corresponding in opacity to that of cholera vaccines of the officially prescribed strength could be obtained by mixing 0.6 ml of the balsam-benzol solution (which was stable) with 9 4 ml of absolute alcohol Results with this method were so satisfactory that it alone was used in the Berlin Gesundheitsamt for the standardization of cholera and typhoid vaccines.

As shown by some other workers, particularly through the exhaustive studies of Brown summarized in his publication in 1919 reliable results in the standardization of bacterial vaccines could be obtained through

method of sterilizing cholera vaccines with phenol alone was afterwards taken by many workers, e g. those in Japan (see Takano and colleagues, 1926) and this procedure is still widely used As quoted by Taylor (1941), the Cholera Advisory Committee of the Indian Research Fund Association recommended in this respect that

"The vibrios should be killed by the addition of 1 per cent phenol to the suspension without the application of heat. The phenol should be reduced to 0.5 / in the vaccine finally issued."

With the exception of formol which as will be noted below has been used to a considerable extent for the sterilization not only of agar grown but also of other types of cholera vaccines, the other chemicals enumerated above have not been used routinely in the manufacture of such vaccines.

(4) *Standardization methods* Introducing the method of cholera vaccination with agar grown vaccines, Kolle (1897) recommended that these ought to contain one "normal" loop or 2 milligrams of fresh culture mass per millilitre To ensure this standard, or that of 2 loops (4 mg) per ml required in Germany at the time of the First World War (see Ditthorn & Loewenthal, 1915 Fischer Bitter & Wagner 1915) some workers merely computed the number of loops by determining the surface area of the media used for cultivation, assuming that one agar slant yielded 10 loops of culture mass a Petri dish of a diameter of 8 8 cm 66 loops, etc. (Soltmann 1915) However this rather crude method of standardization was criticized by other workers, for instance by Ungermann (1917) who pointed out that (*a*) successively prepared media were apt to give different yields, and (*b*) more important still, the abundance of growth depended not merely upon the surface area of the media, but also upon their mass and differed, therefore, according to the thickness of the agar layers. For these reasons the above described method of standardization has been given up in favour of determinations of the bacterial contents of vaccines with the aid of gravimetric or counting procedures, or by opacity tests.

General agreement has been reached that the method of weighing in the course of vaccine manufacture the *moist* culture masses does not yield exact results, mainly because their weight depends upon a large and variable water content rather than upon their bacterial content. As shown by careful observations such as those of Brown (1914 1919) and of Ungermann (1917) determinations of the *dry* weight of the bacterial masses give fully reliable results, but this method is not only rather tedious but of limited value in so far as, whether rightly or wrongly standard values of bacterial vaccines are usually given not in terms of weight but in numbers of the organisms per ml, which have to be determined with the aid of counting methods

As far as the preparation of cholera vaccines is concerned, only two of the latter methods have been used on a large scale namely that introduced

by Wright in 1902 and haemacytometer counts recommended about the same time (see summary by Soltmann, 1915) As is generally known the principle of the former method is to mix equal volumes of the bacterial suspensions to be tested and of fresh human blood in a capillary tube, to prepare stained smears from this mixture and to compare the number of bacteria present with that of the blood corpuscles Since it may be taken that 5 million of the latter are present per ml, this standard value can be used easily to compute the number of organisms per millilitre

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As shown by some other workers particularly through the exhaustive studies of Brown summarized in his publication in 1919 reliable results in the standardization of bacterial vaccines could be obtained through

opacity tests made with the aid of standard barium sulfate suspensions. According to Brown, these quite stable suspensions could be prepared as follows

"A strong solution of barium chloride is made and to this is added an excess of sulphuric acid. The mixture is then boiled and the precipitate is poured on to a filter paper and is washed with tap water until the filtrate is neutral to litmus paper

"The barium sulphate is then dried and thoroughly roasted. When cool a portion is accurately weighed and placed in a perfectly clean mortar The powder is finely ground and the requisite amount of 1 per cent aqueous solution of sodium citrate is gradually added. From this 1 per cent suspension of barium sulphate an 8-fold dilution is made and similarly the other dilutions, the sodium citrate being used throughout as the diluent fluid."

A set of 10 tubes was thus prepared, the first containing the above mentioned eightfold dilution, the second 9 volumes of the same dilution and 1 volume of citrate solution or in comparison to the first tube 90% barium sulfate and so on, the last tube containing 1 volume of the original barium sulfate suspension and 9 volumes of sodium citrate solution.

Brown furnished the following figures showing the relationship of opacity to the weight of dried bacterial substance expressed in milligrams per ml of *V cholerae* suspensions

Percentage of $BaSO_4$ suspensions	*Serial number of opacity tube*	*Weight of vibrios (mg per ml)*	*Numerical equivalent of vibrios (millions)***
100.0	10	2.22	10 926
90.0	9	2.00	9 833
80 0	8	1 80	8 741
70.0	7	1.55	7 648
60.0	6	1.33	6 556
50.0	5	1 11	5 463
40 0	4	0.90	4 370
30.0	3	0 67	3 278
20 0	2	0 44	2 185
10 0	1	0.22	1 093

* As defined in text

** As established with haemacytometer tests by Cunningham & Timothy (1924)

As stated by Gardner (1931) bacterial vaccines may be roughly standardized as follows

"A barium sulphate suspension is made by mixing equal parts of M/100 H_2SO_4 and M/100 $BaCl_2$. This is shaken and distributed into a series of 6 by 5/8 in. test tubes covering the maximum variation in diameter of the stock in use. They are then sealed. The measured bacterial suspension is diluted in a test tube until it is equal in opacity to the standard in a tube of the same diameter The bulk is diluted to the same extent or retained as a known multiple of the standard. Equal opacity is judged by viewing a small luminous flame through the tubes. The opacity will be equal in each if the image is obscured in each at the same distance from the flame."

Joetten (1917) dissatisfied with the exactness of all the above mentioned methods, tested cholera and typhoid vaccines with the aid of complement fixation tests, using the vaccines as antigens and a bacteriolytic cholera immune serum as amboceptor Though he found that vaccines which

appeared to be of uniform value gave identical results in such tests the implementation of this method in the course of mass vaccine production would be fraught with considerable difficulties. The same holds true of the important proposal of Gallut (1949c) to standardize cholera vaccines by determining the weight of the O antigen extracted with the aid of trichlor acetic acid. Gallut maintained in this connexion that only strains with an adequate yield of O antigen equalling at least 5% of the total dry weight of the organisms, should be used for cholera vaccine manufacture

It is important to add that an international reference preparation for opacity tests has recently been introduced by the World Health Organiza tion on the recommendation of its Expert Committee on Biological Standardization this preparation is held for distribution to national laboratories for biological standards by the Statens Seruminstitut, Copenhagen Denmark As stated in a description of the manufacture and properties of this preparation by Maaloe (1955) the material constituting it is a suspension in distilled water of small particles of Pyrex glass as used earlier as a working standard for the characterization of pertussis vaccines and suspensions of challenge bacteria in the Bethesda laboratory of the US Public Health Service Noting that the standard preparation is adjusted to correspond in opacity to a pertussis vaccine with 10 000 million organisms per ml Maaloe emphasized that

"Such translation of opacity into numbers of organisms per ml should, however not be attempted generally It should be stressed that the growth conditions and the subsequent treatment of the organisms making up a vaccine may greatly influence the opacity of the vaccine taken as a function of the number of organisms per ml (this is most pronounced in the case of cholera vaccines) If it is desired, nevertheless, to translate opacity units into number of organisms per ml this number should be determined very carefully on a suspension of bacteria which have been treated in a specified manner The conversion factor obtained for this batch should be used to translate opacity units into number of bacteria per ml *only* when dealing with suspensions of bacteria grown and subsequently treated in this particular manner "

(5) *Dosages* Kolle (1897) finding that a single administration of his vaccine standardized to contain 2 mg of culture mass per ml, produced a satisfactory antibody response in a group of volunteers, maintained that vaccination with such single doses was sufficient for the purposes of cholera control. As noted before actual use of this method was first made during the 1902 epidemic in Japan Reporting on this work, Murata (1904) stated that the dosage recommended by Kolle though giving good results in general, was not invariably sufficient, a number of the vaccinated con tracting the infection. He soon resorted, therefore to single administrations of 1 ml doses of a vaccine of double strength (4 mg of culture mass per ml) and found that none of the persons thus protected contracted cholera.

As has been noted above a standard of 4 mg culture mass per ml of cholera vaccine was also made obligatory in Germany but this vaccine was administered to the armed forces during the First World War in *two*

doses, usually of 0 5 ml and 1 ml, the second injection being given after an interval of 5-7 days According to Takano and co-authors (1926) a two-dose system of cholera vaccination was also soon adopted in Japan where, however 1 ml and 2 ml respectively of a heat killed and phenolized vaccine containing only 2 mg of the vibrios per ml were administered

While the principle that, in order to confer a satisfactory degree of protection against cholera, a two-dose system of vaccination should be adopted has been generally accepted in the experience of many field workers it is often impossible to act accordingly in large-scale vaccination campaigns. This situation has been well described by Russell (1935) who stated that

"The Cholera Commission of the Office International has recently reiterated its view that while vaccination by a single injection is of some value and can be employed in circumstances in which it is the only practicable method, vaccination by two injections should remain the method of choice. In countries like India, however where the number of inoculations to be done urgently may run into many thousands, it is usually impossible to give more than a single inoculation and the common practice is to give a single dose of 1 cc."

It is clear that in order to obtain the best possible results with the system of single-dose vaccination, which had to be adopted for large-scale campaigns not only in India but also in other countries, for instance in China, it is essential to use a high-standard vaccine so as to avoid the administration of too low dosages—a practice unfortunately not rarely resorted to—or to obviate the considerable drawbacks of injecting amounts in excess of 1 ml. This desideratum has been satisfactorily fulfilled in India, where a standard of 8000 million of *V cholerae* per ml has been adopted. In some other countries, however cholera vaccines of lesser strength were used, e g., in China often one of only 2000 million of organisms per ml, administered as a rule in amounts of 1 ml. It was only after Dzen & Yu (1936) had demonstrated the inadequacy of this method through large scale guinea pig experiments, that better counsel prevailed, and Robertson & Pollitzer (1939) working under the auspices of the League of Nations, were able to introduce a vaccine with a vibrio content of 6000 million per ml—a standard afterwards widely adopted in China. However while single-dose administration of this vaccine gave satisfactory results the present writer wishes to emphasize once more that this system of cholera vaccination was adopted merely out of necessity and that it ought to be replaced whenever possible by the administration of two vaccine doses.

(6) *Keeping qualities* As confirmed by numerous observations, for instance by the special studies of Ungermann (1917) killed vaccines, quite particularly cholera vaccines, soon begin to undergo a progressive process of disintegration of the bacterial cells

As maintained by Lange (1922) this process, though often designated as *autolysis* is really one of *cytolysis* and thus fundamentally different from autolysis in the strict sense, due in Lange's opinion to a decomposition of the bacterial protein through the

action of ferments, which had not been destroyed during the process of killing the vaccines by moderate heat nor by the subsequent addition of 0.5 / phenol and were thus able to produce a progressive and thorough reduction of the antigenic properties of the vaccines. Since it was inadvisable to destroy these ferments by the application of higher tempera tures, Lange recommended that formol ought to be used to kill the organisms and to destroy the ferments at the same time. He evidently assumed that autolysis in the strict sense frequently took place in cholera vaccines killed by other methods, but this is not supported by the experiences of other workers quoted below

In contrast to the contentions of Lange, Dold (1925) maintained that less " autolysis " took place in heat killed vaccines than in those sterilized by other methods, both because a kind of protein coagulation took place at temperatures of about 60°C and because such temperatures damaged the autolytic ferments.

A further study of this problem was made by Gohar & Makkawi (1948) Finding that some of the numerous brands of vaccine used for combating the 1947 cholera epidemic in Egypt after a few weeks storage no longer conformed to the prescribed standard of 8000 million per ml, these two workers compared the lysability of cholera vaccines killed and preserved in different ways, making for several days daily counts with a photoelectric colorimeter It was thus established

" that heat-killed vaccines were by far the most stable, apparently because of the adequate coagulation of protein by the heat. Formalin-killed vaccines were also stable but they keep best when preserved with merthiolate instead of phenol. The phenol-killed and preserved vaccines were the least stable."

It is generally held that the cytolysis progressively taking place in killed cholera vaccines does not lead to an accompanying loss of their antigenic properties. Several workers maintained on the contrary that the " opening up " of the bacterial bodies enhanced the efficacy of the vaccines. Be this as it may it is certain that the rapidly commencing process of cytolysis soon leads to changes in the aspect and density of the vaccines, thus render ing them unfit for standardization by opacity tests. Raju (1930) who made a special study of this subject, found that in the course of four weeks the average opacity standards of 29 samples of phenol killed cholera vaccines kept at room temperature (90°F) (32 C) and of 18 such samples kept in cold storage became lowered as follows

Time of testing (days after preparation)	*Average opacity in millions per ml of samples kept at room temperature (90°F) (32°C)*	*in cold storage (32°F) (0°C)*
0 (freshly made)	49 000	49 000
1	29 000	41 000
2	25 000	39 000
3	19 000	38 000
4	18 000	38 000
6	16 000	37 000
14	15 000	36 000
21	13 000	33 000
28	12 000	32 000

doses, usually of 0.5 ml and 1 ml the second injection being given after an interval of 5-7 days. According to Takano and co-authors (1926), a two-dose system of cholera vaccination was also soon adopted in Japan where, however 1 ml and 2 ml respectively of a heat killed and phenolized vaccine containing only 2 mg of the vibrios per ml were administered

While the principle that, in order to confer a satisfactory degree of protection against cholera a two-dose system of vaccination should be adopted has been generally accepted in the experience of many field workers it is often impossible to act accordingly in large-scale vaccination campaigns. This situation has been well described by Russell (1935) who stated that

> The Cholera Commission of the Office International has recently reiterated its view that while vaccination by a single injection is of some value and can be employed in circumstances in which it is the only practicable method, vaccination by two injections should remain the method of choice. In countries like India, however where the number of inoculations to be done urgently may run into many thousands, it is usually impossible to give more than a single inoculation and the common practice is to give a single dose of 1 cc."

It is clear that in order to obtain the best possible results with the system of single-dose vaccination, which had to be adopted for large-scale campaigns not only in India but also in other countries, for instance in China, it is essential to use a high-standard vaccine so as to avoid the administration of too low dosages—a practice unfortunately not rarely resorted to—or to obviate the considerable drawbacks of injecting amounts in excess of 1 ml This desideratum has been satisfactorily fulfilled in India, where a standard of 8000 million of *V cholerae* per ml has been adopted. In some other countries, however cholera vaccines of lesser strength were used, e.g. in China often one of only 2000 million of organisms per ml, administered as a rule in amounts of 1 ml. It was only after Dzen & Yu (1938) had demonstrated the inadequacy of this method through large-scale guinea pig experiments, that better counsel prevailed, and Robertson & Pollitzer (1939) working under the auspices of the League of Nations, were able to introduce a vaccine with a vibrio content of 6000 million per ml—a standard afterwards widely adopted in China. However while single-dose administration of this vaccine gave satisfactory results, the present writer wishes to emphasize once more that this system of cholera vaccination was adopted merely out of necessity and that it ought to be replaced whenever possible by the administration of two vaccine doses.

(6) *Keeping qualities* As confirmed by numerous observations, for instance by the special studies of Ungermann (1917) killed vaccines, quite particularly cholera vaccines soon begin to undergo a progressive process of disintegration of the bacterial cells

As maintained by Lange (1922) this process, though often designated as autolysis, is really one of *cytolysis* and thus fundamentally different from autolysis in the strict sense, due in Lange's opinion to a decomposition of the bacterial protein through the

action of ferments, which had not been destroyed during the process of killing the vaccines by moderate heat nor by the subsequent addition of 0.5 / phenol and were thus able to produce a progressive and thorough reduction of the antigenic properties of the vaccines. Since it was inadvisable to destroy these ferments by the application of higher tempera tures, Lange recommended that formol ought to be used to kill the organisms and to destroy the ferments at the same time. He evidently assumed that autolysis in the strict sense frequently took place in cholera vaccines killed by other methods but this is not supported by the experiences of other workers quoted below

In contrast to the contentions of Lange Dold (1925) maintained that less " autolysis " took place in heat killed vaccines than in those sterilized by other methods, both because a kind of protein coagulation took place at temperatures of about 60°C and because such temperatures damaged the autolytic ferments.

A further study of this problem was made by Gohar & Makkawi (1948) Finding that some of the numerous brands of vaccine used for combating the 1947 cholera epidemic in Egypt after a few weeks storage no longer conformed to the prescribed standard of 8000 million per ml, these two workers compared the lysability of cholera vaccines killed and preserved in different ways, making for several days daily counts with a photoelectric colorimeter It was thus established

" that heat killed vaccines were by far the most stable, apparently because of the adequate coagulation of protein by the heat. Formalin-killed vaccines were also stable but they keep best when preserved with merthiolate instead of phenol. The phenol-killed and preserved vaccines were the least stable."

It is generally held that the cytolysis progressively taking place in killed cholera vaccines does not lead to an accompanying loss of their antigenic properties. Several workers maintained on the contrary that the " opening-up " of the bacterial bodies enhanced the efficacy of the vaccines. Be this as it may it is certain that the rapidly commencing process of cytolysis soon leads to changes in the aspect and density of the vaccines, thus render ing them unfit for standardization by opacity tests. Raju (1930), who made a special study of this subject, found that in the course of four weeks the average opacity standards of 29 samples of phenol-killed cholera vaccines kept at room temperature (90 F) (32 C) and of 18 such samples kept in cold storage became lowered as follows

Time of testing (days after preparation)	*Average opacity in millions per ml of samples kept*	
	at room temperature (90°F) (32°C)	*in cold storage (32°F) (0°C)*
0 (freshly made)	49 000	49 000
1	29 000	41 000
2	25 000	39 000
3	19 000	38 000
4	18 000	38 000
6	16 000	37 000
14	15 000	36 000
21	13 000	33 000
28	12 000	32 000

These observations fully justify the plea of Raju that opacity tests ought to be made on the day of preparation of the vaccines and not a few days afterwards, as it was the practice in some laboratories. Raju added that, as shown by comparative tests, exposure to light exerted no influence on the alterations in opacity but it altered the colour of the samples, giving them a brown tinge

Though the ample observations made in regard to the keeping qualities of cholera vaccines at the time of the First World War in Europe are not always fully comparable in view of differences in the methods of preparation and of testing, it can be gathered that, in the opinion of a majority of the workers there storage for periods of one year or even longer did not cause a loss in potency of the vaccines. Nevertheless, Hetsch (1928) commenting upon these observations, considered it inadvisable to utilize products older than six months to one year He maintained, on the other hand, that cholera vaccines which had been stored for 3-5 weeks produced less marked reactions in man than those used immediately after manufacture

In order to establish whether the formerly adopted policy in India to discountenance the use of cholera vaccines stored for periods longer than six months was justified, Maitra & Ahuja (1932) injected rabbits with a vaccine which had been stored for the period of one year either at 37°C or in a refrigerator at 4 C and determined the agglutinin titres in the sera of these animals. An analysis of the results showed that

"a temperature of 37°C does not cause any appreciable deterioration in the agglutinogenic power of the vaccine. Agglutinin titre of freshly prepared vaccine—administered within a week of its preparation—is about the same as that of one year vaccine stored under above experimental conditions."

Though storage in the refrigerator was found to be more efficient than that in the incubator it appeared unnecessary therefore to take special precautions for the preservation of cholera vaccines for periods of at least one year under temperatures not exceeding 37°C. Under these circumstances, the possibility of an extension of the storage period for such vaccines up to 12 months after their manufacture seemed to deserve serious consideration.

Evidence not only confirming but even amplifying these experiences was obtained by Taylor Ahuja & Singh (1936) through a series of protection tests in guinea pigs with a phenol-killed cholera vaccine, made either soon after manufacture or after storage for various periods It was found that

"vaccines stored up to two years in the plains of India and exposed to hot weather temperature of 111 F [44°C] or over give protection practically equal to freshly made vaccine"

Hence, as far as the knowledge gained through these experiments could be applied to human vaccination, a considerable extension of the six months storage period for cholera vaccines appeared to be permissible even if no cold-storage facilities were available

It is of interest to add that, in order to prolong the storage periods, a few workers such as Chiba (1922) Gutfeld (1922) and Pasricha and co-authors (1941) recommended the use of cholera vaccines which had been exsiccated However in view of the remarkably good keeping qualities of the usual vaccines, there seems to be no urgent call for the manufacture of such dry cholera vaccines

Whole fluid culture (direct) vaccines

Though Brieger & Wassermann demonstrated in 1892 already that administration of broth cultures, which had been killed by heating for 15 minutes at 65 C, rendered guinea pigs immune to infection with virulent cholera vibrios, it was but recently that direct cholera vaccines, prepared from whole fluid cultures were recommended or actually used for human vaccination

Jennings & Linton (1944) obtained by (*a*) cultivating *V cholerae* under continuous aeration with a mixture of air and 20% v/v of CO_2 in a glu cose-containing casein-digest medium and (*b*) sterilizing the growth after an incubation for 24 hours through addition of phenylmercuric nitrate or acetate at the rate of 1 g per litre batch of brew a vaccine with a turbidity of 5 to 10 thousand parts per million of silica and a nitrogen content of about 0 05 g per ml They stated that this product injected subcutaneously in 0 1 and 0 2 ml doses into human volunteers caused no objectionable reactions, but that the serum of these individuals was as efficacious in mouse protection tests as the sera of persons inoculated with a cholera vaccine of the usual type. It has to be noted however, that this claim was not confirmed by Sokhey & Habbu (1950a) according to whose comparative tests the vaccine of Jennings & Linton possessed only 1/20th of the protective power of the casein hydrolysate vaccine described below

As set forth by Sokhey & Habbu (1950a) the shortage of agar supplies in India during the Second World War necessitated a search for a liquid medium suitable for the manufacture of a direct cholera vaccine

A casein hydrolysate medium, prepared according to detailed specifications of Sokhey Habbu & Bharucha (1950) was chosen for this purpose, in which highly virulent cholera vibrios of the Ogawa and Inaba subtypes respectively were cultivated at 37°C for 3 days, 4 ml of 10 / formol were then added per flask of 500 ml to make a strength of 0 08 / and the flasks were again incubated at 37°C for three days. Then, after samples for testing sterility had been withdrawn 15 ml of 0.05 / phenylmercuric nitrate were added per flask as a preservative. The two monovalent vaccines thus prepared were then mixed in equal quantities and put into ampoules.

Sokhey & Habbu considered it indispensable to prepare in this manner a divalent vaccine, because in their experience (see also Sokhey & Habbu, 1950b) there was little cross protection between the Inaba and Ogawa subtypes.

The casein hydrolysate vaccine was standardized by a method of biological assay (Sokhey & Habbu, 1950b) to which further reference will be made below The two workers noted in this connexion that, as shown by haemacytometer counts their vaccine contained not more than 3000 million organisms per ml. Though admitting that this count perhaps did not represent the total bacterial content because vibriolysis was likely to have taken place during the prolonged period of manufacture Sokhey & Habbu drew attention to the possibility that metabolites of *V cholerae* might play a role in immunizing mice

Making comparative tests with their new vaccine on the one hand, with Jennings and Linton s direct cholera vaccine and three samples of agar-grown cholera vaccines manufactured in India on the other hand, Sokhey & Habbu noted that the casein-hydrolysate vaccine had a considerably higher mouse protective power than any of the other products examined. They ascribed the low protective power of the agar-grown vaccines to a lessened virulence of the strains used for their manufacture, but claimed that

" even when virulent strains were used for the preparation of agar-grown vaccines, these were found to have 1/6th to 1/9th of the protective power of casein hydrolysate vaccines made from the same strains "

Though the casein hydrolysate vaccine has been amply used for the purpose of cholera control in India since 1945 with apparently good success, so far no large-scale statistics have become available to assess its value in the field as compared to that of agar grown vaccines. Comparative tests on mice, made in the King Institute, Madras, with (*a*) the agar grown vaccine produced in that institute and (*b*) casein hydrolysate vaccine, failed, according to Pandit (1948) to show a significant difference in the antigenicity of the two products, the animals immunized with either " withstanding approximately 100 times more of the challenge culture than the unprotected mice "

Wahba (1952) compared the efficacy of (1) an agar grown and formol killed vaccine containing 8000 million of cholera vibrios per ml, and (2) a fluid vaccine prepared by growing *V cholerae* in a casamino-acid medium, which, being also formol killed, had a titre of 5000-6000 million organisms per ml Though mouse-protection tests showed no superiority of the fluid vaccine Wahba stressed the simplicity of its manufacture

Comparative tests similar in scope to those described above were made by Ranta & McCreery (1953) with (1) the agar grown and phenol-killed cholera vaccine prepared according to Ranta & Dolman (1943) with a titre of 8000 million vibrios, and (2) a vaccine produced by cultivating the organisms in the chemically defined fluid tyrosine-asparagine-glycine medium of Ranta & McLeod (1950) diluting the growth to contain 8000 million vibrios per ml and adding phenol to a final concentration of 0 5% for the purpose of sterilization. The direct vaccine (2) protected mice

as well as the agar grown product but was found to be less stable because it underwent rapid autolysis Ranta & McCreery suggested, however that it might be possible to overcome this drawback by the judicious use of formol

It is of interest to add that Felsenfeld Young & Ishihara (1950) recommended the sterilization of broth grown cholera vaccines through the action of antibiotics. Neomycin was found to be most suitable for this purpose It deserves attention in this connexion that the avirulent streptomycin resistant cholera vibrios studied by Olitzki & Olitzki (see p 215 above) were found to be fully antigenic and if repeatedly injected capable of protecting mice and guinea pigs against several lethal doses of virulent *V cholerae*

Vaccination with supernatants

Making comparative tests in animals Fairbrother (1928) found that the supernatant fluid of 24-hour-old broth cultures of *V cholerae* obtained through centrifugation possessed but feeble immunizing properties, which were probably due to an incomplete removal of the organisms

This experience was confirmed through further observations in India, Russell (1935) stating in this connexion that a

"study of the immunizing value of cholera vaccines prepared (*a*) from bacterial deposit, (*b*) from the supernatant fluid, and (*c*) from a mixture of deposit and supernatant fluid has revealed that vaccines prepared from supernatant fluid are not only very toxic but possess little protective value. Those prepared from the bacterial deposit are highly protective though slightly less so than vaccines prepared from the whole emulsion."

Vaccination with culture filtrates

As summarized by Hetsch (1912) some early workers first apparently Vincenzi (1892) demonstrated through animal experiments that sterile filtrates of older cholera cultures in broth possessed immunizing properties.

A further noteworthy attempt to produce a cholera vaccine with the aid of candle filtration was made by Strong (1903 1904) who kept for this purpose heat killed suspensions of *V cholerae* in saline for 3 5 days at 37 C before filtering them It was not possible to gather sufficient experience as to the efficacy of this vaccine in the field As shown by tests in laboratory animals and in man the product gave rise to agglutinins and bactericidins, but exerted only a slight antitoxic action

Analogous laboratory experiences were gained by Bertarelli (1905) with a vaccine prepared according to a method similar to that of Strong.

Besredka & Golovanoff (1923) produced a cholera "antivirus" by (*a*) candle filtering cholera cultures which had been incubated for 8-10 days (*b*) reseeding the filtrates with the homologous organisms and again incubating for about 8 days at 37 C and (*c*) filtering once more

When guinea-pigs which had received 1 2 ml of the final filtrate intracutaneously subcutaneously intraperitoneally or intravenously were challenged 24 hours later with 1/10th of a 24-hour-old virulent agar-slant culture of *V cholerae* the majority of the

animals survived. Controls which received in place of the cholera antivirus plain broth or staphylococcus filtrates, succumbed rapidly

The antivirus was found to be heat stable in fact heating for 20 minutes at 100 C seemed to enhance its activity The protection conferred by it, which in the case of intravenous administration was sometimes manifest as early as after 12 hours or even after 6 hours remained complete for 15 days, but then disappeared in the course of the following week.

In a subsequent paper Golovanoff (1924a) reported on comparative tests with the sera of rabbits which had been injected with antivirus preparations and with heat killed cholera vibrios respectively It was found that, though endowed with immunizing properties, the antivirus preparations did not produce agglutinins at titres higher than 1 100

In order to show that the action exerted by the cholera antivirus was of a specific character Golovanoff (1924b) injected groups of guinea pigs with filtrates prepared from *V cholerae* and heterogenous organisms respectively and afterwards challenged all of these animals with lethal doses of cholera vibrios. The filtrates of some of the heterogenous cultures (*Ps pyocyanea, Proteus vulgaris* and *Chromobacterium prodigiosum*) were found to confer an unspecific protection to animals challenged with *V cholerae* after 24 hours, but to none of those challenged 4 days after administration of the heterogenous filtrates. All guinea pigs injected with cholera antivirus survived, regardless of whether they had been challenged after one or four days.

Important immunological studies on cholera filtrates were undertaken by Singer Wei & Hoa (1948a) who either used Seitz filtered cultures of *V cholerae* for this purpose or obtained material from agar cultures by cutting the media extracting the juice by pressure through several layers of cloth, and then centrifuging at high speed before resorting to filtration.

The technique used by these workers to study the activity of the filtrates was as follows

" Guinea-pigs of approximately 300 g weight are bled, the abdomen is opened and the ileo-coecal junction is located. The ileum is excised, freed of mesentery and put into a dish with Tyrode solution. The contents of the ileum are then removed by fitting a syringe into one end of the intestine and rinsing with Tyrode solution. A glass rod of suitable size is passed through the ileum and one end of the intestine is tied to the rod. With a gentle stripping motion the intestine is inverted over the glass rod so that the epithelium faces outwards. The ileum is washed in three changes of Tyrode solution, placed on several layers of thick filter paper which have been soaked with Tyrode solution and cut into pieces of approximately 3 mm in length.

" One half ml portions of the solutions to be tested are placed in test tubes and one piece of ileum is added to each. Tyrode solution is used as diluting fluid. After incubation for one hour in the waterbath the result is read.

" When the reaction is positive the liquid surrounding the intestine becomes turbid and floccules are suspended in it consisting of epithelial cells and mucus which detach themselves from the piece of ileum when the tube is shaken. When the reaction is negative the liquid remains perfectly clear "

Exhaustive studies showed that the "filtrate factor" responsible for the above-described reactions was most regularly produced by cultivation of *V cholerae* in beef-extract broth containing 1% agar only irregularly in digest and peptone water media not at all in synthetic media. It was absent from the saline washings of 24-hour-old agar cultures and the autolysates of cholera vibrios

The filtrate factor was found to be rather heat labile, being almost completely destroyed by exposure to a temperature of 50°C for 30 minutes An untoward influence was also exerted by an acid reaction storage in the incubator for one week, or addition of 0.3% formol, but not by addition of 0.5% phenol.

Discussing the significance of these findings, Singer Wei & Hoa pointed out that the filtrate factor bore in its physical characteristics a close resemblance to a bacterial toxin That according to Burnet and his co-workers (see the preceding chapter) the filtrate factor—called mucinase by them—was a mucin-splitting enzyme was in the opinion of Singer and colleagues not "incompatible with its nature as a toxin as it has become increasingly probable that bacterial toxins have some of the properties of true enzymes" Nevertheless the fact that, as shown by tests with sera raised in rabbits with the aid of H + O and O cholera antigens on the one hand, and with filtrates on the other hand, a close antigenic relationship existed between the filtrate factor and the O antigen of *V cholerae* spoke in the opinion of Singer and colleagues against the former being identical with the cholera toxin. For as they put it,

> "All true bacterial toxins which have been described so far are antigenically specific and entirely different from the somatic antigens of the bacteria by which they are produced"

The conclusion reached by these workers was therefore that antigenically the filtrate factor was very similar to if not identical with, the somatic antigen of *V cholerae*

Considering the practical importance of their findings, Singer, Wei & Hoa stated that

> "The discovery of F F (i.e. the filtrate factor) will not alter the accepted methods of cholera vaccination at present, as the antibodies produced by the somatic antigen in cholera vaccines protect the guinea-pig ileum against the effect of cholera mucinase Preliminary experiments have shown that the sera of human subjects who have been vaccinated with cholera vaccine exert a neutralizing effect similar to the effect of immune rabbit sera."

As stated by Singer and co-authors in a second publication (1948b) they had been able to confirm through further experiments that the sera of vaccinated human subjects as well as cholera immune sera raised in rabbits were able to protect the guinea pig ileum against the effect of cholera filtrates In both instances the protecting antibodies could be removed by absorbing

the sera with boiled suspensions of *V cholerae* The antibodies still demonstrable after absorption being presumably of an anti flagellar character were unable to neutralize the factor responsible for the action of the filtrates

It deserves attention that Singh & Ahuja (1953) to whose observations on the epithelium-desquamating enzyme of vibrios reference has already been made in the third chapter found that strips of intestines freshly dissected from cholera vaccinated guinea pigs were not protected against the desquamating effect of homologous or heterologous vibrio filtrates. These two workers argued, therefore, against the claim of Singer and colleagues that cholera vaccination should confer appreciable protection against the effect of *V cholerae* on the epithelium of the small intestine

An important study of comparative characteristics of variously prepared cholera vaccines with regard to the preservation of mucinase in an antigenic form was recently made by Jensen (1953) For this purpose different vaccines as well as mucinase-containing solutions were used for the immunization of rabbits in order to determine the amounts of antimucinase which might be developed. It was found that immunization with cholera culture filtrates containing mucinase in an active form, gave rise not only to antimucinase at a relatively high titre but also to agglutinins. Administration of filtrates which had been inactivated by heating for 30 minutes at 56 C, while giving rise to the latter antibodies, did not stimulate antimucinase production. Antimucinase was produced only to a low degree in rabbits which had been immunized with washed viable cholera vibrios or with heat phenol or formol killed vaccines.

Since these findings indicated that antimucinase production depended upon the presence of active mucinase in the materials used for immunization, a study of the stability of mucinase under varying conditions was made Jensen established in this respect that

"In a series with varied temperature-time combinations a loss of at least 75 / of the activity was obtained at each of the following points 45 C, 2 hours 37 C, 4 hours 21 C, 48 hours, 4 C, 2 weeks −20 C, 50 / loss in 8 weeks.

"Similar losses within 30 minutes were observed upon the addition of formalin to 0 3 / and of phenol to 0.5 /. With merthiolate added to a final concentration of 0 01 / the losses were no greater than those recorded for preparations without preservative.

"Lyophilization gave preparations which appeared to be quite stable during an 8-week period of testing. The lyophilized material in sealed ampoules withstood the stress of 100°C for 1 hour without loss of activity upon rehydration."

Thus Merthiolate (a proprietary antiseptic known under the international non-proprietary name of thiomersal) seemed a suitable bacteriostatic agent in vaccine manufacture and lyophilization appeared to be useful for the stabilization of the products Further though under experimental conditions immunization with the filtrate factor gave rise to satisfactory agglutinin titres it was in Jensen s opinion desirable nevertheless to use for human

vaccination a killed vaccine to which mucinase had been added To obtain such a vaccine

"a suspension of washed V *cholerae* containing 8×10^9 viable bacteria per ml was mixed with an equal volume of filtrate factor Merthiolate was added to the mixture to a final concentration of 0 01%. This final mixture was then lyophilized the ampoules being finally filled with dry nitrogen and glass-sealed."

Jensen stated that the mucinase titre of this vaccine was 1 1600 and that rehydrated lyophilized material gave the same titre Since there was no detectable loss when the latter material was heated for 1 hour at 100 C, it could be anticipated that this new vaccine would keep well when stored for long periods under lower temperatures. While giving good antibody production in rabbits it appeared to be non toxic for these animals and for mice even upon intracerebral administration

Further studies on the possibility of utilizing mucinase preparations for immunization against cholera were made by Freter (1955), who compared for this purpose the efficacy of (*a*) a killed vaccine obtained by steaming saline suspensions of agar-grown organisms for two hours without pressure and (*b*) a mucinase preparation produced by mixing the thionine glycerol agar used for growing cholera vibrios of the same strain for 16 hours with distilled water emulsifying the mixture and then obtaining with the aid of centrifugation a clear supernatant which was kept for use in lyophilized form. Increasing amounts (0 5 2 ml) of this preparation, standardized to a mucinase titre of 1 128 were used for intraperitoneal injection of guinea pigs which, like those several times inoculated by the same route with the killed vaccine were afterwards challenged through enteric infection with *V cholerae*

As Freter recorded immunization both with the killed vaccine and with the mucinase preparation gave demonstrable protection against cholera infection However he added, in the case of the latter method

"it cannot be decided whether this protection is due to contamination of the mucinase used for immunization with other vibrio antigens or with other enzymes. The low agglutinin titer in protected animals which had been immunized with mucinase as compared to the agglutinin titer of protected animals which had been immunized with boiled vibrios suggests, however that some factor other than agglutinating O-antigen in the mucinase preparations might give effective protection."

It is noteworthy that, as indicated by bacterial counts and mucinase determinations in the intestinal fluids of the animals which had succumbed to the infection neither type of immunization was capable of altering the course of the disease once it had been acquired Likewise the time of death after infection was not different for normal and immunized animals.

As already alluded to in the preceding chapter Lam and co-workers (1955) adduced evidence that cholera mucinase produced a greatly increased permeability of the mouse intestine as manifested by a heightened manometric pressure within the bowel and by an increase in weight of the excised

small intestines indicating a fluid intake into the wall of the gut. Since it was found that passive immunization of the animals against mucinase inhibited the action of the enzyme on the intestine the comparative value of various methods of active immunization was studied, separate groups of animals being given (*a*) killed cholera vaccine only (*b*) mucinase alone, or finally (*c*) a combination of mucinase and vaccine It could be established in this manner that the excised intestines of mice which had been

" actively immunized with *V comma* mucinase and tested with active mucinase gave results somewhat comparable to those obtained with passive immunization, viz., a slight increase in intralumen pressure but to a degree in no way comparable to that obtained with a preparation from an unimmunized animal. A similar result was obtained following immunization with both mucinase and chemically killed *V comma*.

On the other hand, preparations from mice immunized with bacterial cells alone mucinase being absent, showed a heightened sensitivity to mucinase far above that of the non-immunized controls "

Identical results were also obtained in *in vivo* experiments in which mucinase was introduced into the small intestines left *in situ*

An important new method for producing a fluid cholera mucinase preparation which remained stable when combined in various proportions with cholera vaccine has recently been described by Lowenthal (1956) The salient features of processing the mucinase preparation were as follows

Double-strength brain-heart infusion broth was dialysed at 4°C against an equal volume of distilled water for 24 hours this process was repeated three times altogether with fresh distilled water each time. The three resulting dialysate solutions in combination were seeded with 6-hour *V cholerae* cultures grown in the same medium and incubated at 37°C for 17 hours on a roller apparatus in a manner providing maximum aeration without excessive foaming.

After cultivation the bacterial cells were removed by centrifugation followed by Berkefeld filtration, and the filtrate was half-saturated with solid ammonium sulfate. After overnight storage in the cold room, the resulting sediment was dissolved in borate buffered saline until the dialysate no longer precipitated with a saturated barium chloride solution. The product was then sterilized by filtration through a Selas filter

Lowenthal established through appropriate tests that (*a*) the mucinase solution prepared according to his method retained its *in vitro* mucinolytic activity for periods exceeding two years and (*b*) its combination with the cholera vaccine did not affect the *in vitro* activity of the enzyme, the antigenicity of the mucinase or that of the *V cholerae* O antigen. Though admitting that " the role of cholera mucinase has not as yet been definitely established by direct evidence " he maintained that it was advantageous for various reasons to use his purified product in combination with cholera vaccine as an immunizing agent against *V cholerae* infection.

Vaccines prepared from autolysates

As will be gathered from the foregoing section, some workers, particularly Strong, though ultimately resorting to filtration when manufacturing

cholera vaccine, depended in the main upon procedures promoting what is usually called an autolysis of the vibrios Attention has now to be drawn to some further attempts to utilize autolysates of *V cholerae* as vaccines

Gohar (1934) resorted for this purpose to suspensions of cholera vibrios which had been kept in the laboratory for several weeks until all organisms had died and most of them had become lysed Used directly for the immunization of guinea pigs this autolysed vaccine appeared to be more effective than the vaccines killed by exposure to a temperature of 60°C.

Violle (1950) found that the lysate of cholera vibrios which he was able to produce with the aid of supersonic vibration (see Chapter 3 p 161) possessed moderate antigenic properties In his opinion this method would not be suitable for the purpose of practical vaccine manufacture However, the manufacture of a cholera vaccine through application of supersonic vibration has been recorded by Bosco (1955)

Vaccines prepared by extraction methods

Attempts to use extracts prepared in various ways from the organisms for the purposes of cholera vaccination have been made by numerous workers. While as described below most of them resorted to chemical procedures, a few implemented mechanical methods, such as expressing the "plasmatic juice" of previously ground up vibrios (Hahn, 1897) or breaking up the bodies of the organisms at the temperature of liquid air (Macfadyen, 1906) However Hahn s vaccine, though found to confer a long lasting immunity to experimental animals, was apparently never used for human vaccination, while the antigen prepared by Macfadyen served only for serum manufacture

To prepare antigens suitable for cholera immunization by chemical methods, some workers first apparently Klebs (1892) resorted to alcohol extraction. Gohar (1934) used distilled water to extract cholera vibrios which had been dried *in vacuo* To judge from guinea pig experiments, addition of such extracts to heat killed cholera vaccines enhanced the protective power of the latter

Gohar & Isa (1948) stated that they had prepared a soluble extract from cholera vibrios

" by adding to a suspension containing 8 000 million organisms per ml an equal quantity of normal sodium hydroxide and incubating at 37°C. for a few hours until the organisms are dissolved and the suspension becomes clear This is subsequently neutralized with HCl until it is just alkaline (pH about 7.5) "

Fifteen out of 25 rats, which had been twice injected with this extract at a week s interval, survived intraperitoneal challenge with LD_{50} doses of living cholera vibrios.

The possibility of using the nucleoproteids of *V cholerae* referred to above for the purposes of vaccination was experimentally explored by several workers, first by Heller (1905) Schmitz (1906) and Blell (1906)

Though in the opinion of Heller and of Blell nucleoproteids were suitable for human cholera vaccination, Hetsch (1912) stressed on the contrary that, in view of their high toxicity the mediocre titre of the antibodies produced by them, and the technical difficulties of properly manufacturing them, administration of the usual vaccines was far preferable It appears in fact that vaccination with nucleoproteids has never been used for the purpose of protecting man against cholera infection

Toxoids

The use of toxoids prepared from 10-day-old El Tor cultures by addition of 0.5% formol and storage for eight days was recommended for human vaccination against cholera by Kraus & Kovacs (1928) because in their experience (*a*) subcutaneous injection of these products into rabbits and guinea pigs protected these animals not only against one or several lethal doses of the El Tor exotoxin but also against intraperitoneal administration of living El Tor or cholera vibrios, leading as well to agglutinin production in the sera of these animals, and (*b*) the reactions produced by these toxoids in man were not marked.

Felsenfeld & Young (1945) experimenting on rabbits, guinea pigs, and mice with differently prepared cholera vaccines, obtained the best results by using formol- or phenol-killed cholera vibrios of the Inaba subtype combined with formolized filtrates of Inaba or El Tor vibrios. They preferred the latter organisms for preparing such toxoids because these were less toxic and stimulated the production of antibodies against the haemodigestive and necrotoxic action of *V cholerae* to a higher level. The sera of human volunteers tested with this toxoid-vaccine which was used in combination with a dysentery vaccine were found capable of protecting mice against cholera infection.

In order to obtain a cholera toxoid Gohar & Isa (1948) treated the soluble extract they had obtained from suspensions of *V cholerae* through the addition of sodium hydroxide (see page 317) with 0 7% formol and incubated the mixture for 20 days at 37 C While the lethal dose of the original soluble extract for rats was 3 5 ml subcutaneously the formol treated product was almost atoxic and rendered animals immunized with it resistant to large toxin doses Used experimentally for the vaccination of rats which were afterwards challenged with LD_{50} doses of *V cholerae* mixtures of vaccine and toxoid gave better results (84% survival) than toxoid alone (56% survival) the toxic extract or killed vaccines.

Following up preliminary trials by Gohar & Isa, Gohar (1948) experimented with an alum-precipitated cholera endotoxoid prepared by

(*a*) cultivation of *V cholerae* for 2 3 days in broth filled into flat flasks, which were placed horizontally to provide a maximum of aeration (*b*) addition of 0.6% formol followed by further incubation at 37°C for a week (*c*) precipitation with 0.5% alum

and (*d*) collection of the precipitate and resuspension to the required density in phenolized normal saline.

Results obtained by single administration of this vaccine in mice, which were afterwards challenged with LD_{100} doses of living cholera vibrios were almost as good as those produced by two administrations of a heat killed vaccine (40% as against 45% survival)

Tested on a small scale in man the alum-precipitated vaccine produced when injected intradermally indurated masses which persisted for a long time and were in Gohar s opinion thus capable of exerting an antigenic stimulus lasting for several days Administration of full doses subcutaneously produced somewhat severe reactions These observations speak against the practicability of this method of cholera vaccination recommendable though it might be on theoretical grounds

Sensitized vaccine

The use of sensitized cholera vaccines has been recommended by Japanese workers, first apparently in 1916 by Takano and by Yabe (see Shiga, Takano & Yabe, 1918 Takano, Ohtsubo & Inouye, 1926) The method used by Takano for the preparation of such vaccines has been summarized by Takano and colleagues as follows

"A 20-hours agar culture 1 gm is suspended in 2 c.c. of a cholera immune horse serum (bactericidal titre of at least 0 0001 c.c.) diluted four times with the salt solution the mixture is then incubated for 2 hours during which it is shaken from time to time. Then the suspension is centrifuged at very high speed and the organism is washed twice with salt solution. The organism thus sensitized is suspended in physiological salt solution containing 0.5 / of carbolic acid. Of such a suspension 1 c.c. contains 2 mgm. of the organism. The cholera vaccine thus prepared is placed in an incubator overnight An essential part of this process is to prevent the death of the cholera vibrio until the sensitisation is complete. If dead vibrios were to be used, the antigen would be largely set free in the medium and lost in the process of washing, so that the antigenic power of the vaccine would be greatly reduced."

In contrast to these recommendations, a few workers, particularly Besredka (1922) and Masaki (1922a) advocated the use of living sensitized cholera vaccines but these—though found satisfactory in the laboratory—seem not to have been utilized for human vaccination. Sensitized vaccines prepared according to Takano s method have been used on a fairly large scale in Japan with satisfying results, it being claimed in particular that in contrast to the usual type of cholera vaccines the sensitized products caused but mild reactions, yet produced an immunity which set in rapidly and which reached a considerable degree even after single doses only had been administered. Since, however the difficulties of preparing sensitized vaccines on a scale sufficient for mass campaigns are enormous it is not surprising to find that in spite of their undeniable advantages the use of such products has been given up entirely

Cutaneous vaccination

Making parallel observations on guinea pigs which had been injected intraperitoneally at eight days interval with two doses of a heat killed cholera vaccine and on a second group of animals which had the same amounts of vaccine (0.3 and 0 6 ml) rubbed into their freshly-shaven skin Ciuca & Balteanu (1924a) found that the animals of both groups resisted intraperitoneal challenge with a lethal dose of *V cholerae* made 12 days after the second vaccine administration. However it was found that only the intraperitoneally vaccinated guinea pigs and not those protected by the percutaneous route showed agglutinins bacteriolysins and complement fixing antibodies in their sera. Further testing the response to intracutaneous injection of a suspension of live cholera vibrios in 0.2 ml amounts, Ciuca & Balteanu (1924b) noted (*a*) a marked skin reaction in normal guinea pigs (*b*) a less marked reaction in animals protected by two intraperitoneally administered doses of cholera vaccine and tested 12 days after the second injection and (*c*) practically no skin reaction in the animals which had been vaccinated by the percutaneous route Ciuca & Balteanu claimed on account of these findings that percutaneous cholera vaccination produced a local cellular immunity

Interesting as this postulation is it has to be noted that according to all workers who subsequently devoted attention to this point (see Neuhaus & Prausnitz, 1924 for example) intracutaneous administration of cholera vaccines did lead to the appearance of antibodies in the sera of the vaccinated animals. Panja & Das (1947) as well as Singer Wei & Hoa (1948b) who made corresponding observations in man found that antibody formation was apt to take place in the intradermally vaccinated persons to a more marked degree than was the case in subcutaneously vaccinated individuals

Commenting upon their experiences when vaccinating 11 persons intradermally with 0 1 ml and 0.2 ml of the standard Kasauli vaccine and 10 individuals (controls) subcutaneously with 0 5 ml and 1 0 ml doses of the same vaccine Panja & Das lauded the great economy in material effected by the former method and also stated that the reactions produced by intracutaneous vaccination were negligible They admitted, however that in mass campaigns it was considerably less expedient to use the last mentioned in place of the subcutaneous method.

Singer Wei & Hoa (1948b) noted the appearance of small abscesses at the site of the intracutaneous injection in some of the persons vaccinated three times with 0.2 ml doses Since, however such abscesses became manifest only at the third injection, they were probably the result of an allergic reaction and might be avoided by administering only two doses at 5-7 days interval. Be this as it might, no doubt one must share the misgivings of Panja & Das regarding the extrinsic difficulties of using the cutaneous method of cholera vaccination in mass campaigns

Vaccination by the nasal route

Sanarelli (1924b) found that insufflation of a powder consisting of toluene-killed cholera vibrios and boric and lactic acid into the nasal cavity of rabbits led to an often quite considerable formation of agglutinins in the sera of the animals and rendered them immune to cholera infection by the intravenous route

Oral vaccination

While Brieger, Kitasato & Wassermann (1892) reported that they had rendered guinea pigs immune to oral infection with *V cholerae* by intraperitoneal administration of heat killed vaccines, Klemperer (1892) claimed that it was also possible to protect the animals against oral infection by the introduction of small doses of living cholera cultures into their stomach. Similarly Cantacuzène (1894) reported that repeated introduction of cholera vibrios into the stomach of guinea pigs rendered the animals fully resistant to intraperitoneal infection with *V cholerae* provided that they were challenged not earlier than 18 days after the last intragastric inoculation However Sobernheim (1893) though finding that intragastric immunization with rather large doses of cholera vibrios conferred some degree of protection against subsequent intraperitoneal infection, was unable to protect guinea pigs against oral infection either by the intragastric or any other method of cholera vaccination

Pfeiffer & Wassermann (1893) who infected a considerable number of immunized guinea pigs *per os* with recently isolated cholera vibrios found similarly that

"the percentage of immunized guinea-pigs which survive this mode of infection is not appreciably higher than in the control guinea pigs. The mode of immunization seems to exert no influence in this respect. We found no difference when we immunized the guinea-pigs with living or killed cultures, subcutaneously or intraperitoneally when we challenged a few days after immunization or waited for weeks." [Trans.]

Sawtschenko & Sabolotny (Zabolotny) reported in 1893 upon observations they had made when orally administering numerous doses of an agar grown and heat killed cholera vaccine during about a month to themselves and during about two weeks to a student. The total amount of vaccine taken by Sawtschenko was 180 ml, equalling 1 4 g of cholera vibrios weighed in the dry state while Zabolotny ingested 110 ml (dry weight of vibrios about 0 84 g) and the student 135 ml (dry weight of vibrios about 1 g) The sera of Sawtschenko and the student when administered intraperitoneally to guinea pigs in doses of 0 1 1 ml, 25 days after immunization had been completed, protected the animals against challenge with two lethal doses of *V cholerae* made three days afterwards

After they had partaken of further vaccine doses bringing the total dry weight of cholera vibrios administered orally to about 1 7 g and 2.3 g

respectively Sawtschenko & Zabolotny ingested after previous alkaliniza tion of their stomach content 0 1 ml of a 24-hour-old virulent cholera broth culture Though it was possible to demonstrate the presence of the organisms in the stools of Zabolotny up to three days after infection and in the faeces of Sawtschenko on the second day neither of them showed any clinical signs of the disease

Remarkable though these experiences are it has to be pointed out that (*a*) even the attempts to produce cholera artificially through oral infection of non-vaccinated persons were by no means always crowned with success and (*b*) in view of the prolonged course of oral vaccination with enormous doses, the results of the two Russian workers did not furnish any proof of the practicability of this mode of cholera immunization

In a further report made in 1894 Zabolotny stated that he had been able to protect sisels (*Spermophilus guttatus*) against intragastric as well as against intraperitoneal cholera infection through administrations, repeated several times of live attenuated or heat killed *V cholerae* cultures *per os* — results which were afterwards confirmed by Korobkova (1922) Subcutaneous or intraperitoneal immunization of sisels with killed cholera vibrios on the contrary failed to protect the animals against intragastric infection

In analogy with the last mentioned observation Metchnikoff (1894) stressed that he found it impossible to protect young unweaned rabbits by parenteral administration of either living or killed cholera vibrios against oral infection with *V cholerae* which produced in such animals a process apparently identical with that observed in human victims of the disease These negative results were confirmed in 1911 by Choukevitch. As sum marized by Hetsch (1912) analogously disappointing results were also obtained by some other workers with dogs and cats.

The possibility of conferring immunity to cholera through intragastric administration of Galeotti s (1912) nucleoproteid was explored by de Bonis & Natale (1913) They found that only two out of 11 guinea pigs which had been given with the aid of a stomach tube 1 3 doses of 0 005-0.02 g of cholera nucleoproteid dissolved in 0 5% sodium bicarbonate solution survived this treatment They were able to establish, however that (*a*) the sera of 9 of these animals contained agglutinins (maximal titre 1 1000) and (*b*) the two survivors resisted infection with 0 25 ml of a cholera broth culture while a control given the same dose died in 48 hours.

The problem of oral cholera vaccination received far more attention than in the past after Besredka, in a series of articles published in 1918 and 1919 in the *Annales de l'Institut Pasteur* had reported upon successes obtained in the experimental prevention of dysentery typhoid and para typhoid by the combined administration of ox bile and vaccinating doses of the organisms in question by the oral route Masaki (1922b) investigating whether these observations were applicable in the case of cholera, thus summarized the findings he had made in this respect

"(a) Both rabbits and guinea-pigs are completely refractory to the ingestion of cholera vibrios in any dose.

"(b) Oral administration of bile alters the intestinal wall of rabbits, facilitates the entry of the cholera endotoxin and its passage into the system as a consequence agglutinins appear in the animals which had ingested either living or killed vibrios after bile sensitization.

"(c) Ingestion of either living or killed vibrios does engender protective antibodies in sensitized as well as in non-sensitized rabbits.

"(d) Only bile-sensitized rabbits react to the ingestion of living vibrios very high doses (two agar cultures in Roux bottles) kill the animals in one to two weeks median doses (one culture) render the animals ill for some days doses less than half a Roux bottle finally cause no harm.

"(e) Only bile-sensitized animals which have shown illness after the ingestion of living vibrios, become vaccinated against intravenous administration of a surely lethal dose of vibrios." [Trans.]

Masaki added that the immunity engendered in this manner was in all probability a local (intestinal) one. In this connexion, he laid stress upon the fact that, though ingestion of bile followed by that of living cholera vibrios led to the appearance of agglutinins in the sera of the rabbits these antibodies, instead of augmenting, decreased and finally disappeared, apparently because administration of the initial vibrio doses had led to a "vaccination" of the intestinal wall which thus became impermeable to the organisms or their products

The validity of Masaki s conclusions was supported by some laboratory observations e g. those of Glotoff (1923) and of Horowitz Wlassowa & Pirojnikova (1926) but some other workers such as Sdrosdowsky (Sdrodowski) (1924) and Kilchin & Vigodtschikoff (1925) took a definite stand against the method of oral cholera vaccination Engelhardt & Ray (1927) concluded from an exhaustive study that it was possible to immunize bile sensitized rabbits by oral administration of very large doses of *living* cholera vibrios against intravenous infection The agglutinin titre in the sera of these animals rose but slightly and then decreased. In the rabbits which were orally given *killed* cholera vibrios after bile administration, no immunity resulted but the agglutinin titre of their sera rose constantly during immunization and the following two weeks

In view of these discrepant results it is not surprising to find that the opinions held by the different observers in regard to the question of whether cholera immunization *per os* led to a local or a general immunity, were rather divided Some, for instance Horowitz Wlassowa & Pirojnikova were in favour of the former view but others Sdrosdowsky for example denied the existence of a separate enteric immunity of histogenous origin —an opinion also vigorously expressed by Hetsch (1928)

On account of the observations he had made in the past with Sawtschenko Zabolotny (1922) advocated the large scale use of oral

vaccination for coping with the cholera situation in Russia. As summarized in the *Tropical Diseases Bulletin* (1923) he

"recommends the use of vaccines prepared from thick suspensions of organisms killed by heat, carbolic acid or alcohol (20-40 per cent.) from 3 to 5 doses of 2 10 c.c. every other day Each dose contains from 10 to 100 milliard vibrios, or from 0 01 to 0.1 gm. of dried organisms. Vaccines were also prepared in the form of tablets with sugar or cocoa, each containing 0 1 gm. of dried organisms."

Zabolotny stated that as shown by preliminary experiences, in persons who had been immunized against cholera in this manner the agglutinin titre rose to 1 400 and the bactericidal titre to 1 60 His article does not indicate whether large scale advantage of oral cholera vaccination was taken in Russia.

Among the studies made during the years following Zabolotny's publication in regard to the appearance of antibodies in the sera of individuals orally vaccinated against cholera, the following deserve mention

Korobkova & Zénine (1923) tested the sera of 49 out of 348 persons who had been immunized by oral administration on each of 3 subsequent days of one tablet respectively containing 50 milliards (US billions) of heat-killed cholera vibrios, 115 of these individuals also receiving on each occasion a bile tablet. In the sera of 19 individuals, which were examined 17 days after immunization, an agglutinin titre of 1 100 (the maximum tested) was found to be present invariably regardless of whether or not bile tablets had been given.

In 30 persons, whose sera were tested 5 weeks after immunization, agglutinins were but rarely demonstrable, but bacteriolysins at titres ranging from 1 10 to 1 25 were found to be present with one exception, both in the group receiving vaccine only and in that receiving vaccine and bile tablets In Korobkova's opinion it was therefore uncertain whether bile administration had to be combined with the oral administration of cholera vaccines. The same doubt was also expressed by Peverelli (1924).

Gluchow and co-workers (1923) testing the sera of 73 individuals vaccinated orally noted the appearance of agglutinins and bacteriolysins which persisted for 9 months, but decreased in the majority 4 months after vaccination to half the titre. A repetition of oral vaccination did not lead to an increased antibody titre, but such an increase was noted after the subcutaneous administration of booster doses. In the opinion of the above mentioned workers these observations supported the view that oral vaccination created a barrier against the passage of vibrios or their products through the intestinal mucosa, which could be circumvented through parenteral revaccination. This view was opposed by Stepanoff-Grigoreff & Iljina (1924) in whose opinion the appearance of agglutinins in practically all persons orally vaccinated and that of bacteriolysins in part of these individuals manifested the development of a general immunity

Far more important than the experiences recorded above were large-scale trials of the method of oral cholera vaccination in India,[1] made simultaneously with mass vaccinations by the subcutaneous route

The procedure adopted in these campaigns for oral vaccination was to administer on each of three consecutive mornings, before food had been taken, first a bile tablet and 15 minutes later a commercially prepared bilivaccin tablet with a bacterial content of

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[1] As reported by Sarramon (1930) comparative study of oral and parenteral cholera vaccination was also made in Indochina. It showed attack rates of 0 36 % in the 4982 persons who had received bilivaccin and of 0.37% in 8485 individuals vaccinated parenterally as against attack rates of 2.02% and 1.67% respectively in the two control groups of 11 004 and 29 254 persons

about 70 milliards (billions) of dried cholera vibrios. Parenteral vaccination consisted of the subcutaneous injection of either one or two doses of a standard cholera vaccine with a vibrio content of 8000 million

Reporting on the first of these trials, Russell (1928a, 1928b) submitted the following figures

		Cholera attacks	Cholera deaths	Percentage attacked	Percentage mortality among attacked
A *Bilivaccin*					
Number given 3 doses of bilivaccin	4 982	18	4	0.36	22.2
Number not treated (controls)	11 004	222	93	2.02	41 9
B. *Cholera vaccine*					
Number of persons given one dose (0.5 ml)	17 160	59*	25*	0.34	37.3
Number of persons given two doses (1.5 ml)	8 485	31	2	0.37	6.5
Number of persons not treated (controls)	25 645	489	184	1 67	37 6

* Attacks and deaths occurring within three days after vaccination excluded.

While these figures, besides illustrating the value of the usual method of cholera vaccination even with a single 0.5-ml dose demonstrate also the efficacy of oral vaccination, it has to be noted that in the experience of the field staff

" the bilivaccin sometimes produced acute diarrhoea of such a severe type that the persons affected refused to take further doses, and, in certain cases indeed, the medical officers were accused of inducing cholera. Fortunately no untoward incident occurred as those affected quickly recovered."

Submitting the gross figures quoted above and the subsidiary statistics to a painstaking analysis Russell reached the conclusion that

" [*a*] the immunity developed five days after a single dose of anti-cholera vaccine is nearly as high as that conferred three days after a full course of oral bilivaccin

" [*b*] It may be inferred that a high degree of immunity is conferred by both the subcutaneous anti-cholera vaccine and the oral bilivaccin, but that the former is, in the long run, superior to the latter In view of the fact that, with ordinary precautions, the risk of injury from inoculation is inappreciable and that even transitory discomfort is uncommon, the case in favour of anti-cholera vaccine as a practical and cheap preventive measure is complete."

As stated by Russell (1935) during 1932 another large field experiment with bilivaccin and with anti-cholera vaccine was carried out in endemic cholera areas of Madras Presidency (now Madras State) several thousand persons being protected by the former method and an additional 6000 persons by the latter The conclusions reached after statistical analysis were that

" (1) both the full three-dose course of bilivaccine and the 1 c.c. dose of anti-cholera vaccine conferred a considerable degree of protection against cholera and

"(2) the incidence of cholera amongst the unprotected was 8.5 times higher than among those protected by bilivaccine (3 doses) and 5.5 times higher than amongst those protected by anti-cholera vaccine."

In Russell's opinion these observations seemed to confirm those made in the first field study He added

"The question of the substitution of bilivaccine for anti-cholera vaccine for the protection of Haj pilgrims was recently referred to the Office International for an expression of opinion, but that body has declared that while vaccination *per os* probably produces a certain immunity this is much inferior to that obtained by subcutaneous inoculation. Moreover the difficulty of exercising a strict control appeared to the Office International to be sufficient reason for rejecting the suggestion."

There can be no doubt that the almost insurmountable difficulties of properly using oral cholera vaccination under the conditions ordinarily prevailing during mass campaigns as well as the unpleasant and sometimes even serious reactions apt to follow bile administration strongly speak in favour of parenteral immunization It was probably for these reasons as well as on account of the difficulty and costliness of preparing oral vaccines that after having received much attention for some time the method of cholera vaccination *per os* has been given up entirely

Mixed vaccines

Castellani, who seems to have been the first worker to draw attention to the possibility of using mixtures of vaccines manufactured individually from different bacterial species for simultaneous immunization against the respective infections suggested in 1913 that combined administrations might be made of vaccines prepared from live attenuated cholera vibrios and dysentery bacilli He and Mendelson (1915) followed this proposal by recommending the use of a tetra vaccine obtained by mixing vaccines prepared separately from typhoid, paratyphoid A and B bacilli, and from cholera vibrios, the finished product containing per ml 500 million of the first mentioned organisms, 250 million each of the two paratyphoid strains and 1000 million of *V cholerae* It is of interest that 0.5% phenol alone was used to sterilize these vaccines, a storage of the phenolized suspensions at 10 20°C for a few hours being found sufficient for this purpose Dealing exhaustively with the use of various combined vaccines, Castellani (1916) recommended *inter alia* a mixed cholera and plague vaccine and a "penta vaccine" for simultaneous immunization not only against these two infections but also against typhoid and paratyphoid A and B

As summarized by Hetsch (1928) numerous European workers recommended and to some extent practised combined vaccinations against typhoid and cholera at the time of the First World War while in the Philippines Manalang (1925) made ample use of single-dose administration of a tetra vaccine prepared according to Castellani's method, which contained

per ml 4000 million cholera vibrios 2000 million typhoid bacilli and 1000 million each of paratyphoid bacilli A and B

More recently Gefen (1945) recommended a polyvalent vaccine prepared with the aid of extraction methods for simultaneous vaccination against cholera, typhoid, paratyphoid dysentery and tetanus Similarly Ranta & Dolman (1943) recorded favourable results of laboratory tests (production of agglutinins in rabbits) with a combined vaccine containing 4000 million of cholera vibrios 700 million of typhoid bacilli 225 million respectively of paratyphoid bacilli A and B per ml of tetanus toxoid Felsenfeld & Young (1945) to whose method of manufacturing a toxoid vaccine against cholera reference has been made above tested a mixture of this and a similarly prepared dysentery vaccine on a group of volunteers and obtained satisfactory results when using the sera of these individuals for serological and mouse protection tests

While in some instances the combined vaccines were issued ready made by the manufacturing laboratories some workers mixed the individual vaccines they proposed to administer in combination immediately before use Schwarz (1919) was not satisfied with either of these procedures fearing on the one hand that storage of combined vaccines might lead to a deterioration of their immunizing power and, on the other hand, that an instantaneous mixture of individual vaccines might lead to contaminations He advocated, therefore mixing the vaccines destined for combined administration two days before their use so that the phenol or other antiseptic contained in them could cope with contaminating organisms.

Judging from experiences gained through laboratory tests mainly those with the sera of persons to whom the combined vaccines had been administered, the various workers using such products were unanimous in asserting their efficacy Indeed some observers, e g. Manalang (1925) maintained that the combination of different vaccines exerted a stimulating influence on the immunizing power of the individual components It was also generally held that the reactions caused by the administration of mixed vaccines were not more marked than those following the separate use of the single components of these products However fairly ample experience of combined cholera and typhoid paratyphoid vaccination in China has convinced the present writer that the latter claim holds true only to a limited extent while the reactions produced by these mixed vaccines were not more marked than those caused by the administration of typhoid-paratyphoid vaccines alone they were much stormier than those resulting from the sole use of cholera vaccines Hence while the absence of marked reactions was instrumental in overcoming the prejudice against sole cholera vaccination the use of the combined vaccines invariably led to complaints seriously hampering the campaigns In the considered opinion of the present writer it is not advisable therefore to use mixed vaccines in the course of general anti-cholera campaigns the less so because as a rule these have been

prepared with substandard amounts of cholera vibrios—a drawback which becomes particularly serious when only single vaccine doses can be administered. One must admit, however that under special conditions, particularly when applying vaccination methods for the protection of armed forces, the advisability of using combined vaccines of an adequate standard deserves consideration.

Negative phase

As in the case of other infectious diseases, so also in that of cholera it has been asserted by some workers that vaccination is followed by a negative phase during which the susceptibility of the immunized to the infection is temporarily increased. As can be gathered from a study of the literature, particularly the statements of Pfeiffer & Friedberger (1908b) Aaser (1910) Bessau & Paetsch (1912) Papamarku (1917) Schwartz (1919) and Hetsch (1928) these claims were mainly based on the one hand on observations of changes in the titre of immune bodies in the sera of the vaccinated and on the other hand on experiences regarding the incidence of cholera in recently immunized individuals

The claims made by some observers that a negative phase was created through a drop in the antibody content of the sera of the vaccinated deserve no credence not only because such a lowering of the antibody titres has not been confirmed by other workers but also because the laboratory experiences discussed below clearly show that cholera vaccination is not followed by a phase of temporarily increased susceptibility to the infection

Exhaustive studies made in this respect by Pfeiffer & Friedberger (1908b) showed that experimental animals which had been immunized with specific vaccines, instead of becoming increasingly susceptible to cholera during the period following immediately showed on the contrary at once a resistance—probably at first an unspecific resistance—to the infection Bessau & Paetsch (1912) continuing these studies were also unable to demonstrate the presence of a negative phase through animal experiments performed " under conditions which had to be considered very favourable in comparison to human immunization " Attention has been drawn already (see page 251) to further observations by Papamarku (1917) who showed that the drop in bactericidal power observable in the sera of guinea pigs after cholera revaccination was as a rule not accompanied by a loss of resistance to challenge infection.

When it is considered that (*a*) as generally agreed, a period of at least about three days has to elapse before the immunity engendered by active cholera immunization begins to become manifest, and (*b*) during outbreaks it is inevitable that some persons, because they are incubating the disease at the time of vaccination, fall ill before being protected, it is easy to under stand why as soon as Haffkine s method of vaccination began to be prac tised, its many adversaries clamoured that this procedure instead of pre-

venting, caused or at least facilitated the appearance of cholera. Haffkine took a determined stand against the idea of a negative phase stating for instance in a speech given in 1899 before the Royal Society London in reference to some of his early statistics that

"Inoculation has again acted, so to say immediately or as we have adopted to generally formulate the result, has acted within the time necessary for the subsidence of the general reactionary symptoms produced by the inoculation."

In spite of these and other reassurances, the idea of a negative phase following cholera vaccination in man continued to be ventilated from time to time for instance as late as in 1950 by Dani However a large majority of the cholera workers considered the appearance of the disease in quite recently vaccinated persons merely a *post hoc* and not a *propter hoc* phenomenon Thus Simpson (1915) referring to careful observations made in this respect in India denied that vaccination against cholera led to a negative phase in man and his opinion has been endorsed by many workers dealing with this infection in Central Europe during the First World War Petrovich (1915) even stated that he had used cholera vaccine with good success for the treatment of a quite considerable number of patients who had been attacked by the disease It was also pointed out with much reason by several workers (see Hetsch, 1928) that the non appearance of clinical signs of the disease in specifically vaccinated carriers of *V cholerae* strongly spoke against the appearance of a negative phase More important still, a large-scale statistical study by Adiseshan Pandit & Venkatraman (1947) to which full attention will be paid in the tenth chapter failed to show that the administration of a standard cholera vaccine in single doses led to a significantly increased incidence of the disease among the inoculated.

The evidence adduced above suffices to show that one should not hesitate in emergencies to make ample use of cholera vaccination even during epidemics At the same time it is clear however that both in order to benefit as many persons as possible and to avoid alarming the people by the occurrence of the disease in recently vaccinated individuals every possible effort should be made to administer the vaccinations before onset of the cholera seasons

Duration of immunity

While as discussed above general agreement exists that active cholera immunization does not confer protection against the infection during the days immediately following administration of the vaccine and it is also usually held that a period of about a week has to elapse before a substantial immunity becomes established, opinions regarding the duration of the immunity vary Haffkine, summarizing the experiences with his vaccines up to 1906 maintained in this connexion that

"their effect becomes rapidly accentuated during the first few days and lasts, when moderate doses are used, for about 14 months, after which time it begins to decrease markedly and probably to disappear"

Though some workers stated that cholera vaccines manufactured according to Kolle s method conferred immunity for a year others main tained that the period of protection afforded by killed cholera vaccines lasted only for 7 9 months, or merely for 6 months or even less Adiseshan Pandit & Venkatraman (1947) summarizing the observations they were able to make in the course of their above mentioned statistical study, stated in this connexion that

" In the villages which had second outbreaks within the first six months after the first outbreak the incidence of cholera in persons who had been inoculated at the time of the first outbreak is definitely lower than in the uninoculated. These differences are statistically significant. The protection afforded by anticholera inoculation continues, therefore, for at least six months.

" Although the figures available are too small to warrant a definite conclusion, the virtual absence of cholera among inoculated persons in villages which were re-infected between six and twelve months after the first outbreak suggests that immunity may last for as long as twelve months."

It follows from these observations that in localities were cholera becomes, or is apt to become epidemic perennially at least yearly revaccinations are indicated. Hetsch (1928) summarizing the experience acquired re garding the duration of the immunity after cholera vaccination during the First World War was of the opinion that as long as a danger of infection continued to exist, 0 5-ml booster doses of cholera vaccines ought to be administered at half yearly intervals—a procedure found satisfactory in the German and Austrian armies

Evaluating tests

It is essential to state that the tests used to assay the immunogenic value of bacterial vaccines in general, and of cholera vaccine in particular form only part of the examinations necessary to ascertain the suitability of these products It is of prime importance to determine on the one hand that the finished products are of proper standard and to ascertain on the other that they are free from aerobic or anaerobic contamination and, in the case of killed vaccines, also that the method of sterilization used has been effective Unless it is incumbent upon a laboratory to assay com mercially produced vaccines, the implementation of the tests just mentioned forms a part of the routine followed for vaccine manufacture

A further preliminary step of great importance is to ascertain that the cholera vaccines issued produce no undue reaction on account of either an exalted toxicity or of too high a content in phenol or other antiseptics Pasricha, Chatterjee & Paul (1938) recommended that the absence of an unduly high toxicity be proved by the survival of guinea pigs given 5-ml doses of the products under test intraperitoneally and that the absence of an excess of antiseptics be demonstrated by showing that adult mice which had been subcutaneously injected with 0 5 ml doses of the vaccines examined remained free from serious symptoms during the period of one week.

The important methods available in practice for assessing the immunizing properties of cholera vaccines may thus be classified

(1) direct agglutination tests with the vaccines as antigens and standard cholera immune sera,

(2) serological tests (including Pfeiffer tests) to ascertain the presence of antibodies in the sera of actively immunized animals or vaccinated human subjects

(3) active immunization experiments in laboratory animals

(4) protection tests with the sera of vaccinated animals or human subjects.

The importance of these various methods will now be dealt with seriatim

(1) *Direct agglutination tests* Summarizing the experiences of Pasricha and co-workers (1938 1941) when examining numerous cholera vaccines of different origin Taylor (1941) stated that these workers took advantage of three categories of tests, namely (*a*) direct agglutination of the finished vaccines with pure O sera of the Inaba and Ogawa subtypes (*b*) agglutinogenic tests in rabbits and (*c*) protection tests in guinea pigs Taylor emphasized that

"The results of these tests were found to run parallel to each other and when a vaccine did not agglutinate to satisfactory titre with the O sera no protection against an infecting dose of *V cholerae* was obtained."

Endorsing on account of these experiences the great value of direct agglutination tests Taylor concluded therefore that "if a vaccine is sterile and shows satisfactory agglutination it can be considered satisfactory"

(2) *Serological tests* As has been stated before, agglutination tests with the sera of immunized animals and more still with those of vaccinated human beings have been made by numerous workers but have yielded rather discrepant results. There can be no doubt that besides showing up differences in the character of the various vaccines examined, these discrepant results were due to a large extent to extrinsic causes, particularly (*a*) differences in the size and number of the vaccine doses administered, and (*b*) differences in the technique implemented by the various workers especially the use of less suitable killed antigens in place of live cholera vibrios At the same time it must be admitted, however that even if the tests are performed in an adequate manner with the sera of suitably vaccinated individuals agglutinins if at all demonstrable appear at different and not rarely rather insignificant titres and persist for different periods, not necessarily coinciding in length with those during which immunity is supposed to last

While in the opinion of some workers these inconstancies sufficed to render agglutination tests with the sera of vaccinated subjects of little value for the assay of cholera vaccines other observers made a far more

Though some workers stated that cholera vaccines manufactured according to Kolle s method conferred immunity for a year others maintained that the period of protection afforded by killed cholera vaccines lasted only for 7 9 months or merely for 6 months or even less Adiseshan Pandit & Venkatraman (1947) summarizing the observations they were able to make in the course of their above mentioned statistical study stated *in this connexion that*

"In the villages which had second outbreaks within the first six months after the first outbreak, the incidence of cholera in persons who had been inoculated at the time of the first outbreak is definitely lower than in the uninoculated. These differences are statistically significant. The protection afforded by anticholera inoculation continues, therefore, for at least six months.

"Although the figures available are too small to warrant a definite conclusion, the virtual absence of cholera among inoculated persons in villages which were re-infected between six and twelve months after the first outbreak suggests that immunity may last for as long as twelve months."

It follows from these observations that in localities were cholera becomes, or is apt to become epidemic perennially at least yearly revaccinations are indicated Hetsch (1928) summarizing the experience acquired regarding the duration of the immunity after cholera vaccination during the First World War was of the opinion that as long as a danger of infection continued to exist, 0 5-ml booster doses of cholera vaccines ought to be administered at half yearly intervals—a procedure found satisfactory in the German and Austrian armies

Evaluating tests

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examination and then challenging the animals thus protected with doses of *V. cholerae* found to be lethal for controls, which already guided Ferrán and Haffkine, has been continuously used by subsequent workers.

While at first exclusive use of guinea pigs was made for this purpose other species of experimental animals have been preferred by most of the recent workers. As noted before (see pages 317 and 318) Gohar & Isa (1948) in order to compare the efficacy of various cholera vaccines resorted to active immunization tests with rats. White mice seem first to have been used for this purpose by Fennel (1919) to assess the efficacy of a cholera lipovaccine he had manufactured. However, large-scale use of these animals for the assay of cholera vaccines became possible only when Griffitts (1942) showed that suitably small yet highly virulent test doses for the intraperitoneal challenge of mice could be obtained by suspending the cholera vibrios chosen for this purpose in 5% mucin instead of in normal saline. It is of historical interest to note in this connexion the previous observation of Cantacuzène & Marie (1919a) that otherwise sublethal doses of *V. cholerae* to which extracts from the small intestine or caecum of guinea pigs had been added in small quantities proved fatal if intraperitoneally administered to animals of this species.

Taking advantage of Griffitts' findings the National Health Institute at Washington recommended tentatively in 1942 a mouse protection test for the assay of cholera vaccines. As summarized by Ranta & Dolman (1943) this method

"involves vaccinating each of a group of at least 30 white mice, about five weeks old and weighing 8-10 gm., with a single intraperitoneal dose of the test vaccine, equivalent to about 400 million vibrios. An equal number of similar mice is set aside at the outset for control purposes. Fourteen days later one-half of the mice in both the vaccinated and non-vaccinated groups are given intraperitoneally approximately 500 000 live vibrios of a virulent Inaba-strain suspended in mucin, while the remainder receive similar doses of a virulent Ogawa type strain. The requirement is that at least 50 per cent of the mice in each vaccinated group should survive for 72 hours, while at least 75% of the non-vaccinated mice should die of cholera septicaemia within 72 hours."

Ranta & Dolman (1943) confirmed both the advantage of using suspensions of *V. cholerae* in 5% mucin for the challenge of immunized mice and the value of mouse protection tests for the assay of cholera vaccines. They recommended in 1944 a modification of such tests based upon the use of two spaced doses of the vaccines under examination and requiring survival of 100% of groups of not less than 15 mice challenged with 5 MLD of mucinized vibrios, and of at least 80% of batches of such animals challenged with 10 MLD. They considered it unnecessary to challenge the animals with both Inaba and Ogawa strains as had been recommended by the National Institute of Health, because in their experience there existed a cross-protection between these two subtypes.

The problems involved in the mouse protection tests were exhaustively studied by Burrows et al. (1947) who reached the conclusion that

serious objection, namely that the results of such tests merely demonstrated the antigenicity instead of the immunizing power of the vaccines examined. Since however in the experience of many workers a considerable parallelism existed between the results of agglutination tests and those obtained with active immunization of test animals one should not be rash in denying the value of the former far more expedient, method It also deserves great attention that, as indicated by the studies of Burrows et al (1947) possibly together with other antibodies O agglutinins do play a role in the protection against cholera infection

It is generally acknowledged that the demonstration of bacteriolysins in the sera of specifically vaccinated experimental animals is indicative of a state of immunity against parenteral infection with *V cholerae* Since however a fundamental difference exists between the morbid process produced in this manner under experimental conditions and the disease spontaneously developing in man after the ingestion of cholera-contaminated materials, it is difficult to decide whether or to what extent the presence of bacteriolysins in the sera of cholera vaccinated human subjects testifies to the existence of an immunity against natural infection with *V cholerae* Even some of the workers who were agreed that cholera vaccination is apt to protect man against such an infection, ascribed little importance to the presence of bacteriolysins in the sera of the vaccinated. For instance, Papamarku (1917) maintained in this connexion that these bodies may be absent or present at low titres only in the sera at times when the individuals in question are supposedly still protected against cholera infection by the previous vaccination

However while one must admit that the presence of bacteriolysins in the sera of cholera vaccinated individuals furnishes no direct proof of the existence of an immunity against the infection bactericidal tests with such sera are of value in so far as a considerable degree of parallelism has been found to exist between the results they yield and those of active and passive immunization tests The technical difficulties attendant upon the bactericidal tests and their consequent tediousness render them less practicable in routine work than the above-evaluated agglutination tests While therefore the latter seem to be preferable it has to be kept in mind that no close parallelism has been found to exist between the results yielded respectively by these two serological methods It also deserves attention that in the experience of Ahuja & Singh (1948) the outcome of bactericidal tests alone compared favourably with that of passive mouse-protection tests

Complement fixation tests with the sera of the vaccinated, while presenting considerable technical difficulties, seem to possess no superior value in comparison with the two serological methods discussed above

(3) *Active immunization tests* The method of testing cholera vaccines by actively immunizing experimental animals with the products under

"In our 1893 memoir we referred to observations made in three persons, two of whom had been vaccinated by Haffkine, whereas the third served as control All three showed the same symptoms of benign cholera which one observes in the majority of cholera infections in the laboratory Ferrán himself as well as some of the individuals vaccinated by him suffered after ingestion of cholera vibrios from diarrhoea like the non-vaccinated. His co-worker Pauli had choleraic diarrhoea even though he had received 13 vaccine injections. Zlatogoroff (1904) ., though vaccinated four times with killed and living cholera vibrios, suffered after ingestion o. a cholera culture from diarrhoea and was obliged to take calomel after he had a third fluid stool" [Trans.]

It might be argued that the cholera attacks in the above mentioned vaccinated subjects were invariably slight, but one must fully agree with Metchnikoff that the attempts to induce cholera artificially in man gave rather inconstant results in non vaccinated as well as in immunized individuals, producing often only slight symptoms, if any at all

However in marked contrast to the above mentioned failures or uncertainties, recently Burrows & Ware (1953) obtained impressive results when immunizing guinea pigs intraperitoneally with three doses of cholera O vaccine and afterwards challenging the animals by the intragastric route As summarized by the two workers

"Active immunization with cholera O vaccine results in a 14-fold increase in the ID_{50} dose (median infective dose) at the height of the immune response, 4 days after a course of vaccine, which declines to 8 7-fold at 14 days and 1 9-fold at 28 days."

These results as well as previous observations of Burrows and co-workers on the appearance of antibodies in the faeces of actively cholera *immunized guinea pigs and human volunteers which will be discussed later*, seem to endorse the value of active immunization tests for an assay of cholera vaccines

(4) *Passive protection tests* A passive mouse protection test for assaying the results of cholera vaccination has been recommended by Griffitts (1944) As summarized by Burrows et al. (1947) this worker when reporting the results of titration of protective antibodies in the sera of immunized human volunteers

"expressed the titer in two ways, the number of LD_{50} doses protected against by 0 1 ml of serum, and the amount of serum required to protect 50 / of the mice receiving various doses of vibrios. By the first method, normal serum showed a titer of less than 3,000 and the immune serum of 100,000 to 200 000 By the second, 0 1 ml of normal serum did not protect against 590,000 vibrios, and of pooled immune serum, 0 068 ml protected 50 / of mice receiving 59 million vibrios, 0 01 ml 50 / of those receiving 5.9 million, and 0.0014 ml those receiving 590 000 vibrios."

Burrows et al. (1947) using the sera of 25 cholera immunized volunteers for passive mouse protection tests formed a most unfavourable opinion on the value of this method However Ahuja & Singh (1948) using guinea pigs for passive as well as for active protection tests reached the conclusion that the former represented "the most sensitive method available in the

"A standard dose or fold increase method of titration of protective antibody was found to be impractical, but protective titer expressed as the ratio of the LD_{50} dose for immune mice to that for control mice was reproducible within reasonable limits, and the results were comparable provided that the virulence of challenge strains was substantially the same. The variability was such that 100 fold differences in titer were regarded as significant, 10 to 100 fold suggestive, and 10 fold or less as not significant."

In a further study on the biological assay of cholera vaccines Sokhey & Habbu (1950b) pointed out that the mouse protection test suggested by Ranta & Dolman (1944) was not sufficiently exact because it used too large vaccine doses for the immunization of the animals It was for this reason that Ranta & Dolman postulated the existence of a cross protection between the Inaba and Ogawa subtypes which Sokhey & Habbu were unable to confirm.

The principle of a new method for the biological assay of cholera vaccines introduced by the two last mentioned workers which gave reproducible results within narrow limits, was to determine "the dose of vaccine required to protect 50% of the immunized animals against a challenge dose constant both in numbers and virulence and producing 100% mortality among the controls"

As noted before Sokhey & Habbu preserved suitably virulent strains for challenging their animals by freeze-drying. After regeneration the selected strain was grown for three hours in nutrient broth and one part of a 10^{-3} dilution of this was added to four parts of a 5% mucin suspension 0.5 ml of this mixture which was used for intraperitoneal injection of the test animals, contained about 100 000 organisms and represented 100 times the minimum lethal dose

Though, as will be discussed below some modern observers are inclined to place more reliance upon passive protection tests there can be no doubt that properly conducted active immunization tests yield fully reliable results as far as the degree of immunity conferred by parenteral administration of the vaccines in question against parenteral infection with test doses of *V cholerae* is concerned. It is clear however that the results of such tests even if most favourable furnish no direct answer to the question to what extent parenteral administration of the vaccines concerned is apt to protect man against oral cholera infection.

In order to obtain such final proof it would be necessary to demonstrate that the vaccines in question, if subcutaneously administered, are capable of protecting the vaccinated animals or human subjects against oral cholera infection As has been discussed before (see pages 321 322) experiments made in this respect by some earlier workers not only failed to furnish such final proof in a convincing manner but gave as a rule frankly negative results A few analogous trials made in man likewise proved disappointing. Metchnikoff (1911) summarizing the results of such attempts, stated that

school were not shared by some other workers, who explained the mechanism of active cholera immunity in different ways

Most noteworthy in this connexion are the views of Metchnikoff (1895) and some other French observers (see Hetsch 1912) according to whom not a humoral immunity depending upon the action of bactericidal substances but the phagocytic activity of the leucocytes took the decisive part in the destruction of the cholera vibrios in the bodies of immunized animals However Pfeiffer (1894b) quoting experimental observations according to which vibriolysis took place in the peritoneal cavity of cholera immunized guinea pigs without any marked participation of leucocytes, maintained that phagocytosis instead of being of prime importance in the process of cholera immunity was an accompanying phenomenon (*Begleiterscheinung*) of a secondary character—an opinion which appears to have been shared by most subsequent workers

Another noteworthy objection to the views of Pfeiffer and colleagues was made by Gruber (1896) in whose opinion agglutination of the causative organisms was of primary importance in the process of cholera immunity the agglomerated vibrios then becoming amenable to the action of protective substances present in the bodies not only of immunized but also of normal animals.

However as summarized by Sobernheim (1897) and by Hetsch (1912) Gruber s theory was not in accord with the observations of several other workers. Pfeiffer & Kolle (1896) stressed in this connexion that the phenomenon of agglutination observable *in vitro* represented merely a passing stage, after which the vibrios again became capable of multiplication More important still they as well as other workers showed that cholera immune sera, including the sera of convalescents, even though they had become devoid of agglutinating properties for various reasons could still exert a bacteriolytic action in the animal body It also deserved great attention that as demonstrated for instance by Kolle (1901) different methods of immunization led to differences in the antibody content of the resulting sera, agglutinins appearing rapidly and to a high titre in the sera of intravenously immunized animals in particular whereas subcutaneous or intraperitoneal administration of *V cholerae* led to the production of prevalently bacteriolytic sera. Considering these and analogous experiences Hetsch (1912) concluded that

"the specific bacteriolysins of R. Pfeiffer and the agglutinins of Gruber Durham are different substances occurring side by side in the cholera-immune serum. The agglutinins can be considered as the result of a reaction of the organism to the infection and to some extent also as indicators of an immunity The typical bacteriolysis, however is produced solely through the bactericidal substances of R. Pfeiffer" [Trans.]

However while feeling convinced of the paramount importance of the bacteriolysins in the protection of immunized guinea pigs against infection with *V cholerae* Pfeiffer & Wassermann (1893) warned against using these

present state of our knowledge for demonstrating differences in the immunizing value of vibrio strains"

As has been noted before Ahuja & Singh considered bactericidal tests fairly trustworthy because they gave a response approximately parallel to the results of passive protection tests They were on the contrary not favourably impressed by the utility of agglutination tests with the sera of vaccinated subjects for an assessment of the value of cholera immunization.

Mechanism of active cholera immunity

As pointed out by Pfeiffer & Wassermann (1893) in a classical study on the mechanism (*Wesen*) of the active immunity against cholera, the fact that immunized guinea pigs resisted challenge with larger amounts of living cholera vibrios than non-immune animals could be interpreted by assuming that "immunization might have conferred either *antitoxic* or *bactericidal* properties"

Exhaustively investigating which of these two factors was at work, Pfeiffer & Wassermann found that guinea pigs, regardless of whether they had been immunized with live or killed vibrios, by the subcutaneous or the intraperitoneal route were practically as susceptible to intraperitoneal challenge with killed cholera vibrios as non-immune animals. It was clear therefore that the immunized animals had not acquired a resistance against the cholera toxin (*Giftfestigkeit*) On the other hand, it could be shown that living cholera vibrios injected into the peritoneal cavity of immunized guinea pigs perished there far more rapidly than was the case in normal animals Even if immunized guinea pigs succumbed to challenge infection because they had been injected with overwhelming doses of *V cholerae* their peritoneal cavity was as a rule sterile. The conclusion reached by Pfeiffer & Wassermann on account of these experiments as well as of passive immunization tests which will be discussed later was that

"It was erroneous to consider cholera immunity as a resistance against the toxin [*Giftfestigung*], as has been invariably done in the previous publications. In active as well as in passive immunization there develop exclusively bactericidal properties" [Trans.]

An identical conclusion was reached independently by Sobernheim (1893) who stated that

"substances must have been produced in the blood of [cholera] immunized animals which impede the development of living bacteria and, therefore, the production of the lethal amount of toxin, but which do not interfere with the deleterious action of the already formed toxin. Consequently the animals are immune in the strict sense, but not resistant to the toxin [*giftfest*]!" [Trans.]

As can be gathered from a study of the literature, particularly from a valuable summary by Hetsch (1912) the views of Pfeiffer & Wassermann and of Sobernheim, though soon adopted as the creed of the official German

Attention has first to be drawn in the latter connexion to observations made by Cantacuzène (1894) Cantacuzène & Marie (1919a, 1919b) and Inouye (1928)

Cantacuzène (1894) noted that cholera vibrios which had been introduced by the intragastric route into subcutaneously or intraperitoneally vaccinated guinea-pigs dis appeared from the small intestine after 3 hours, whereas they persisted there abundantly for 24 hours in non-vaccinated animals He postulated, therefore that a "bactericidal *milieu*" existed in the small intestine of cholera vaccinated guinea-pigs.

As already alluded to (page 333) Cantacuzène & Marie (1919a) found that extracts prepared from the small intestine of guinea-pigs by mincing, drying *in vacuo* suspension in normal saline storage in the refrigerator for 24-48 hours, centrifugation filtration through paper and inactivation by exposure to 56 C for ½ hour if added in quantities of 0.5 ml or 1 ml to a non-lethal dose of cholera vibrios, rendered the latter rapidly fatal for intraperitoneally infected guinea-pigs. This "activating" property was manifested not only by extracts obtained from the intestines of normal guinea-pigs, but to an even more marked degree by those derived from cholera-vaccinated animals. However the extracts obtained from the latter category of animals were found to protect guinea-pigs against intraperitoneal injection with lethal cholera doses if administered subcutaneously 6 hours before infection.

Supplementing these observations by complement-fixation tests with intestinal extracts prepared in the manner described above, Cantacuzène & Marie (1919b) found that

(*a*) The complement-fixing properties of extracts from the intestines of normal guinea-pigs were variable but as a rule not marked.

(*b*) On the contrary the extracts obtained from the small intestines of guinea-pigs which had either received 24 hours previously a lethal dose of *V cholerae* intragastrically or had been injected intraperitoneally 24 or 72 hours previously with heat killed cholera vibrios, showed most marked complement-fixing properties. The extract from the caecum of these animals gave much feebler reactions, their blood sera quite feeble reactions or even none at all.

(*c*) The extracts from the small intestines of solidly cholera vaccinated guinea-pigs as well as their sera showed most marked complement-fixing properties.

Inouye (1928) working with extracts prepared like those of Cantacuzène & Marie but usually without resorting to desiccation, found that

(*a*) guinea-pigs which were intraperitoneally injected with intestinal extracts from animals vaccinated subcutaneously resisted an immediately following challenge with lethal doses of *V cholerae* whereas guinea pigs previously injected intraperitoneally with intestinal extracts from non-vaccinated animals succumbed even to non-lethal cholera doses

(*b*) such non-lethal doses proved also fatal to animals which were given intraperitoneally extracts prepared from the liver or kidneys or muscles of normal guinea-pigs, in a solitary experience also to an animal injected intraperitoneally with the intestinal extract of an orally vaccinated guinea-pig

(*c*) on the contrary extracts from the liver or the spleen of parenterally vaccinated guinea-pigs did not exert a sensitizing action, thus not causing the death of animals challenged with sublethal doses of *V cholerae*

Though in view of the method of experimentation chosen by Inouye the value of his results appears to be limited, it is noteworthy that the extracts prepared from the intestines of parenterally vaccinated animals protected guinea pigs against lethal cholera doses

"theoretically interesting facts" for an explanation of the immunity against human cholera, because as they stressed, the latter was

"absolutely different from the mixed process of infection and intoxication which is produced in guinea pigs through intraperitoneal injection of cholera bacteria" [Trans.]

In order to study in which organs of the animal body the cholera immune bodies were formed, Pfeiffer & Marx (1898) subcutaneously injected strong young rabbits with agar slant doses of heat killed cholera vibrios and then determined the bacteriolytic titres of their sera and leucocytes as well as of extracts of their organs, prepared by (*a*) triturating weighed quantities with the aid of glass powder (*b*) mixing the triturates with measured amounts of broth and (*c*) removing the glass particles by centrifugation after one day s storage in the refrigerator

While obtaining no evidence that the leucocytes served "as the matrix or even as the vehicles of the cholera immune bodies" Pfeiffer & Marx found as summarized by Hetsch (1912)

"when examining after different intervals the extracts of the various organs for their antibody content that, hand in hand with a rapid increase of the immunity in certain organs a considerably higher quantity of bacteriolysins was demonstrable than in the circulating blood. This held true in the first place of the spleen and the bone-marrow next of the lymph-nodes and the lungs. Unexpectedly it was further found that in the majority of the experiments the spleen contained already during the second day after the vaccination clearly demonstrable amounts of cholera immune bodies, even when hardly any traces of such were recognizable in the blood serum." [Trans]

In the opinion of Pfeiffer & Marx, the abundance (*Plus*) of the immune bodies found in the spleen, bone marrow and the lymph nodes indicated that a rapid production of these substances took place there, which was in excess of their secretion into the blood stream Apparently this excess of immune bodies in the above-mentioned organs gradually decreased and was no more manifest when immunity had become maximal While these findings referred in the first place to the bacteriolysins Pfeiffer & Marx obtained some evidence to show that the agglutinins behaved in an identical manner

On account of these and related observations Pfeiffer & Marx felt convinced that the production of cholera immune bodies took place in the blood forming (*blutbereitenden*) organs, the spleen the bone-marrow and the lymph-nodes

This conclusion of Pfeiffer & Marx, being in accord with the now generally accepted concept that the reticulo-endothelial cells, found in organs like the spleen, the bone marrow the lymph nodes, and the liver are the site of production of the immune bodies, continues to be considered valid However a considerable debate arose over the question whether a production of cholera immune bodies took place in the intestine

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Cantacuzène (1920) discussing the findings made by him and Marie felt convinced that the general immunity produced by parenteral cholera vaccination was accompanied, or rather preceded by a rapidly appearing local immunity Judging from ample and favourable experiences in the Romanian army during the Balkan wars and during the First World War he considered parenteral cholera vaccination which in his opinion conferred an antibacterial immunity as an "absolutely rational" method

As has been stated already some of the advocates of oral cholera vaccination, such as Masaki (1922) maintained that this mode of immunization led to a local intestinal immunity but one must fully agree with Hetsch (1928) that the evidence brought forward in this respect was by no means convincing, whereas not only other observations made in the case of cholera (see Wassermann & Sommerfeld, 1915 for example) but also ample evidence adduced in the case of other bacterial infections spoke against this concept In order to support his view Hetsch quoted the following conclusion reached by Neufeld (1924)

"There exist markedly different degrees of active immunity but no different kinds, as if for instance through preliminary treatment with living cultures another kind of immunity would be produced as after the injection of killed cultures, or after oral administration of bacteria one different from that following subcutaneous administration or as if natural recovery from an (infectious) disease would necessarily [*grundsätzlich*] lead to a state of immunity different from that following artificial immunization. The new knowledge on the formation of antibodies supports the basic concepts of Ehrlich, even though his ideas have to be modified in a few respects [Trans.]

Attention has now to be paid to recent exhaustive studies by Burrows and his co-workers, which shed new light on the mechanism of active immunity against cholera.

Taking advantage of the new methods for the isolation and purification of the endotoxin of *V cholerae* introduced by Burrows (1944) he and his co-workers studied first the immunological properties of this endotoxin (see Burrows et al 1944) The main results of this investigation were that

(*a*) An immunological, hapten-like activity of cholera endotoxin prepared by preliminary extraction with alcohol and three subsequent precipitations with chilled acetone was indicated by skin reactions in immune rabbits as well as by specific precipitation and complement-fixation produced by rabbit immune sera.

(*b*) In a series of rabbit immunization tests with differently prepared types of endotoxin most remarkable results were obtained with dialysates, which in the case of animals immunized in 5 or 6 doses with a total of 7.5-8.5 mg led to a protective titre of at least 100 000 as well as to a marked agglutinin response (titres 1 50 000 or more).

Thus, as measured by the agglutinin response, these dialysates possessed antigenic properties markedly superior to those of whole vibrios. Similarly mice could be actively immunized by three intraperitoneal inoculations of an alcohol-saline suspension of alcoholic toxin with doses far below those needed to afford the same degree of protection with heat-killed cholera vaccines.

(*c*) It was not possible, however to demonstrate an *in vitro* neutralization of the activity of the endotoxin preparations by either antibacterial or anti-endotoxic sera or to

immunize mice either actively or passively against the lethal effect of intraperitoneal administration of the purified cholera endotoxin.

Studying the permeability of the small intestine of rabbits and guinea pigs *in vitro* Burrows Wagner & Mather (1944) found that addition of living vibrios or of crude or purified cholera endotoxin to the Ringer Locke solutions used for these tests markedly accelerated the rate of flow through strips of normal intestine If however, in place of these strips of intestine from immune animals were used

"they were completely or almost completely resistant to the action of the toxin and in its presence showed little or no difference in permeability to fluids from normal intestine in the absence of toxin"

As summarized by Burrows and his co-workers these findings, which were consistent with and complementary to those recorded in the first paper by Burrows et al (1944) indicated that

"active immunity to Asiatic cholera in the experimental animal, and presumably also in man, includes antitoxic as well as antibacterial immunity"

It deserves attention, however that according to further experiments recorded by Burrows (1953) the immunity resulting from active immunization with purified cholera endotoxin proved to be inferior to that produced by active immunization with O antigen.

Reporting upon observations made in guinea pigs which had been infected with *V cholerae* by the intragastric route following alkalinization and intraperitoneal administration of opium tincture, Burrows Elliott & Havens (1947) stated that

(*a*) the infection thus produced was confined essentially to the lumen of the bowel with no consistent or significant spread into the tissues and organs, and could be considered to be of a true nature in view of an enormous multiplication of the vibrios in the intestine of the animals

(*b*) prior administration of a non-lethal dose of *V cholerae per os* or intraperitoneal immunization with 2 mg of cholera O vaccine two weeks before challenge protected the animals against as much as three lethal doses and altered the pattern of vibrio excretion characteristic of non-immunized infected animals by bringing about a sharp reduction in the number of organisms, especially early in the infection, and usually leading to a lessened persistence of the infection

(*c*) antibody activity manifested by the appearance of agglutinins and protective antibodies, and shown to be due to the presence of immuno-globulin, was demonstrated in the faeces of immunized animals and also in the faeces of human volunteers who had been vaccinated against cholera

(*d*) the antibody in the faeces, called *coproantibody* to distinguish it from that in the serum, though appearing early and reaching peak titres before antibodies became manifest in the serum, disappeared in contrast to the serum antibodies in 3-4 weeks

The correlation found to exist between the pattern of vibrio excretion characteristic for cholera immunized animals and the presence of copro antibody led to the conclusion that "effective immunity to enteric infection is associated with pre-existing coproantibody"

As shown by further studies of Burrows & Havens (1948) immune globulin, immunologically indistinguishable from immune-serum globulin was excreted in the faeces and urine of actively or passively cholera immunized guinea pigs as well as of cholera vaccinated human volunteers It was established in this connexion that the barrier between the tissues and the lumen of the bowel was readily permeable to the immune globulin, which thus could pass in either direction

Burrows & Havens confirmed that there was a lag between the appearance of peak agglutinin titres in the faeces and urine of immunized guinea pigs and in their sera respectively This lag was still more conspicuous in cholera vaccinated human subjects in whom the peak of the faecal agglutinin titre was reached about two weeks after the second inoculation, and that in the urine apparently a few days later as compared to the occurrence of peak titres in the serum after 30 to 42 days There was also a difference in the disappearance of the antibodies, the agglutinins demonstrable in the urine and faeces falling to insignificant levels in the case of immunized guinea pigs after 3-4 weeks in that of human subjects two to three months after vaccination, whereas—as far as could be ascertained—the antibodies persisted in the sera for considerably longer periods

It could not be established definitely where the faecal antibodies were formed Considering various possibilities, Burrows & Havens stated that

"It is possible that fecal antibody is that which is formed locally The assumption that, with the general antigenic stimulus of parenteral inoculation, the antibody-forming cells of the intestine form antibody to excess much more rapidly than those of for example, the spleen, seems unlikely and is without foundation. The corollary assumption, that the association of falling fecal titer and rising serum titer may be explained by an earlier cessation of antibody formation by the local cells or a breakdown of the mechanism of diffusion or secretion, is hardly tenable especially in view of the results of experiments reported here in which fecal titer was maintained by periodic reinoculation. Neither of these need be made, however for it is possible that the observed independence of serum and fecal antibody titers is a concentration effect. Thus, the dilution of immune globulin liberated by antibody-forming cells during the early stages of immunization is relatively much greater in the body fluids than in the feces, but antibody accumulates in the tissues and not in the feces. Rising serum antibody would, then, represent a rate of accumulation of serum globulin while the titer of fecal antibody would reflect more directly its rate of diffusion from the antibody-forming cells."

The findings made in regard to the coproantibody were confirmed by some further investigations of Burrows and co-workers, particularly by the results of a study on the quantitative relationship between the faecal and serum antibodies made with the aid of complement fixation tests by Koshland & Burrows (1950)

It would be rash to assert that the observations of Burrows and colleagues, the main features of which alone could receive attention within the scope of the present disquisition, have fully solved the problems of the mechanism of active cholera immunity In fact, they have furnished reason

to assume that as Burrows et al (1947) put it when commenting upon the mouse protection test

"It may well be that protection is a manifestation of more than one kind of anti body possibly O agglutinin bacteriolysin, immune opsonin and the like as well as antibody to the vibrio endotoxin at least present evidence does not justify the assumption that protective antibody is homogeneous.

There can be no doubt however that the demonstration of a local response to parenteral cholera immunization in the intestine, manifested by the rapid appearance of coproantibody, goes a long way to support the assumption that this method of immunization is an effective means to protect man against natural infection with *V cholerae*

Passive Immunity

Observations which demonstrated the possibility of conferring a passive immunity against cholera seem to have been recorded first in 1892 by Gamaleia, Klemperer and Lazarus

Gamaleia reported at a meeting of the Société de Biologie, Paris, (October 29 1892) that Ketscher in St. Petersburg had (*a*) found that the milk of cholera-immunized goats, if intraperitoneally administered to guinea-pigs in 5-ml doses, protected the latter animals against lethal doses of *V cholerae* given intraperitoneally or intramuscularly and (*b*) noted even the survival of guinea-pigs which had received intraperitoneal injections of such milk after they had been infected with cholera vibrios by the intramuscular or intra peritoneal route.

Klemperer (1892c) besides confirming that the milk of immunized goats was capable of protecting guinea pigs against intraperitoneal cholera infection, also found the same to hold true of the serum of a human volunteer who had been injected with 5 ml of milk from a cholera immunized goat. Klemperer (1892b) further established that the serum of persons vaccinated with living or heat killed cholera vibrios protected guinea-pigs against intraperitoneal injections of *V cholerae*

As already alluded to Lazarus (1892) found that the serum of cholera convalescents, if administered in extremely small doses (minimum one decimilligram, 0 0001 g) intra peritoneally to guinea pigs, protected the animals against intraperitoneal challenge with lethal doses of *V cholerae* made a few hours afterwards. He established, on the other hand, that even enormous doses of cholera convalescent serum were incapable of saving previously cholera infected guinea-pigs which already showed signs of illness. Lazarus maintained, therefore, with great reason that

"in the case of animals which it was possible to save from death through treatment [with convalescent serum] following infection, we cannot speak of a successful cure but rather of an immunization administered during the incubation stage" [Trans.]

As can be gathered from a study of the literature (see summaries by Hetsch, 1912, and Kraus 1929) during the years following these initial findings the question whether passive immunization against cholera conferred an antitoxic or merely a bactericidal immunity became the subject of considerable debate Though, as will be shown later this con troversy is now of historical interest rather than of actual importance the

following of the several claims made for the production of cholera-antitoxic sera (see in addition to the summaries just mentioned those of Hetsch, 1928 Harvey 1929 and of Burrows et al. 1944) deserve attention

Ransom (1895 see also Ransom & Kitashima, 1898) immunized goats and horses with a soluble cholera toxin which he claimed to have obtained by heating 5- to 10-day-old broth cultures of *V cholerae* for a short time at 100 C, filtering through a Pukal filter and then resorting to concentration *in vacuo* and alcohol precipitation at 30°C. As admitted by Ransom himself (1898) his sera possessed feeble (*schwache*) antitoxic properties. Pfeiffer (1895) even maintained that the antitoxic effects of Ransom s serum were not higher than those of normal sera.

Metchnikoff and co-workers (1896) prepared cholera sera by immunizing goats and horses as well as guinea-pigs and rabbits with what they considered a soluble toxin of *V cholerae* This thermostable product was obtained from strains the toxigenicity of which had been enhanced through passage in collodion bags kept in the peritoneal cavity of guinea-pigs. The vibrios in question were afterwards grown in a medium containing besides 2 / peptone, 2 / gelatin, and 1 / sodium chloride, also 10 / of normal horse serum for 4 days (when toxicity was found to be maximal) and then filtered.

The sera obtained with this toxic product through prolonged immunization of horses had marked bactericidal and agglutinating properties. At the same time they were endowed with what Salimbeni (1908) considered a somewhat feeble antitoxic power at least 1 ml being needed to protect a guinea-pig against 4 lethal toxin doses. Nevertheless, as summarized by Salimbeni (1908) " the serum proved to be very efficacious prophylactically and gave in curative tests very good results in intestinal cholera of young rabbits produced experimentally according to Metchnikoff's method "

Salimbeni (1908) continuing the above-described work, modified the methods originally adopted by Metchnikoff and collaborators (1896) by (*a*) ceasing to use the preliminary passage of the strains in collodion bags through guinea-pigs, which was found to be unnecessary when freshly isolated human cholera strains, never passed through animals and kept with rare transplantations on agar at room temperature, were available (*b*) increasing the content of horse serum in the medium used for toxin production to 25 and (*c*) adopting in place of the subcutaneous the intravenous route of immunization. He noted in this connexion that, while 1.5 ml of serum from a subcutaneously immunized horse had been needed to neutralize 4 lethal doses of cholera toxin, after continued intravenous immunization of the same horse $^1/_3$ ml of its serum sufficed for this purpose

It is noteworthy that prolonged intravenous immunization of a horse with living cholera vibrios (saline suspensions of agar cultures) also gave a serum which combined marked agglutinating and bactericidal properties with a quite marked power to neutralize the toxin. This was in contrast to the results of intraperitoneal immunization with living cholera vibrios attempted by Metchnikoff and collaborators (1896) the serum obtained in this manner from a horse, though endowed with marked agglutinating and bactericidal properties, showing less antitoxic power since 1 5 ml were necessary to neutralize two lethal toxin doses.

As alluded to before (page 317) Macfadyen resorted, in order to liberate the cholera endotoxin, to the method of fragmentating growths of *V cholerae* obtained through cultivation on agar for 18 hours, at the temperature of liquid air The thermolabile endotoxic products obtained in this manner were capable of producing sera which possessed, besides agglutinating and bacteriolytic properties, anti-endotoxic power For instance, the serum of an intravenously immunized goat was found to neutralize in guinea-pig tests 8 lethal endotoxin doses, whereas normal goat serum proved incapable of protecting these animals even against two lethal doses.

Brau & Denier (1906) in order to obtain toxic products for the manufacture of cholera immune sera, (*a*) grew a strain of *V cholerae* isolated in Saigon, which was found to con

gulate milk rapidly but was non-haemolytic, in a medium consisting of 90 / normal horse serum and 10 / defibrinated horse blood, and (*b*) resorted after an incubation at 39 C for 7 days to filtration first through paper and then through Chamberland or Berkefeld filters.

Summarizing their experiences when using sera produced with the aid of this toxic filtrate and, for the sake of comparison, also a serum raised by Salimbeni against live cholera vibrios, Brau & Denier stated that

"(*a*) Subcutaneous injection of the toxin into goats, rabbits, guinea-pigs and horses produces with difficulty an active immunity The serum thus obtained is feebly antitoxic.

"(*b*) Intravenous injection on the contrary immunizes the animals and leads to the appearance of very manifest antitoxic properties in their serum.

"(*c*) The animals which had been intravenously injected with living cultures furnish a more active serum than those treated with the soluble toxins" [Trans.]

Brau & Denier maintained, therefore with much reason that there seemed no need to "establish a difference between the cholera toxin contained in the bodies of the microbes and that obtained in the culture fluid"

It is important to add that (*a*) the serum produced by Brau & Denier had also quite good agglutinating properties (titre 1 5000) (*b*) their toxin, the production of which they ascribed to a maceration of the vibrios in the course of cultivation was found to be thermostable, a serum raised with a filtrate which had been heated for 20 minutes at 100 C proving as efficacious as those prepared with unheated toxic filtrates, and (*c*) Brau & Denier established that neutralization of lethal doses of their toxin by graduated serum doses did not take place according to the law of multiple proportions, as is characteristic of true bacterial exotoxins.

Kraus, summarizing in 1909 the results obtained in the production of antitoxic cholera sera (see Kraus, 1907 Kraus & Russ, 1908) stated "that not only the cholera vibrio but also the El Tor vibrios and many other vibrios have the property to produce toxins (*Gifte vom Charakter der als Toxine charakterisierten Gifte*) and that these can be neutralized with specific antitoxins" Therefore, in order to produce antitoxic sera, Kraus and Kraus & Russ used not only true *V cholerae* strains, but also El Tor strains as well as the *V Nasik* a haemolytic vibrio not reacted upon by cholera-immune sera.

The sera raised with the toxins, i.e., the filtrates of 6- to 8-day-old broth cultures of true cholera vibrios, though also producing bacteriolysis of El Tor vibrios, exerted an antitoxic action only against the *V cholerae* toxins The El Tor serum, while bacteriolytic not only for the El Tor but also for classical cholera vibrios, was found to be antitoxic not only in the case of these two organisms but also of cholera-like vibrios Serum obtained with *V Nasik* toxins, while bacteriolytic for this organism only proved antitoxic also for El Tor vibrios

While finding guinea-pigs rather unsuitable for tests with these sera (see below), Kraus reported that

"1 Mice which are infected with cholera vibrios are cured if one treats them either with antitoxic cholera serum or with serum obtained with the toxin of specific El Tor vibrios (i.e., El Tor vibrios in the strict sense) Sera obtained with *V Nasik* toxins were inefficacious in this case.

2 Mice infected with specific El Tor vibrios are cured it treated with sera prepared with El Tor or Nasik toxins. Cholera serum produced in horses failed completely [but, as stated by Kraus in a footnote a cholera serum produced by Pfeiffer in goats neutralized also El Tor toxin].

3 Mice infected with *V Nasik* are cured if they are treated with sera prepared with El Tor or Nasik toxin Cholera serum exerts no curative action." [Trans.]

On account of these findings Kraus stressed that success in cholera treatment could be obtained only with those sera which contained antitoxins as well as bacteriolysins. It has to be noted, however that the mice used in such tests could be saved only if treated with the appropriate sera not later than one hour after infection. Guinea-pigs could be saved but occasionally if given large serum doses intravenously half an hour after infection. Kraus warned, therefore, against concluding from favourable results obtained in one animal species upon the possible effect of the sera in other species and expressed at the same time the fear that man might react in respect to specific cholera treatment like the guinea pig and not like the mouse.

Schurupow (1909) reporting upon tests with one of Kraus cholera sera, which had been sent to Russia, stated that this serum possessed but minimal antitoxic properties and produced agglutination of cholera vibrios only at a titre of 1 500.

In order to prepare a cholera serum of his own, Schurupow tried first to immunize horses with living cholera vibrios (route not stated). However the serum of these animals was found to possess only marked agglutinating and bactericidal properties but no antitoxic power Schurupow admitted, however that these experiences were not in accord with those of some other workers, Kraus for instance having obtained through subcutaneous immunization of horses with living cholera vibrios a serum, 0.07-0.1 ml of which neutralized 1 ml of toxin

In view of the disappointing results he had obtained with live cholera vibrios, Schurupow immunized horses with Chamberland-candle filtrates of *V cholerae* cultures which had been treated with alkali. The resulting sera possessed agglutinating properties (titre 1 10 000), but had according to Schurupow no bactericidal power As far as can be gathered from the somewhat scanty data furnished by this worker his sera showed quite considerable protective and even curative action against the endotoxin, but unless quite high doses were administered prophylactically the guinea-pigs tested showed marked signs of intoxication before eventually recovering.

As will be gathered from the above summary the various workers enumerated, though using different antigens for the immunization of their animals unanimously stated that they had obtained sera more or less endowed with antitoxic properties The validity of these claims was, however vigorously opposed by Pfeiffer and his co-workers

Pfeiffer & Wassermann (1893) making passive immunization tests in guinea pigs with cholera convalescent sera, found ample evidence of a bactericidal action of these sera, but none of a role of antitoxins, and concluded, therefore that passive as well as active cholera immunity depended upon the presence of bacteriolysins This contention was supported by Issaeff (1894) who found that the sera of cholera immunized guinea pigs as well as those of human convalescents though endowed with marked protective and to some extent even with curative properties, possessed no antitoxic properties, the maximal cholera toxin doses tolerated by immunized guinea pigs being not higher than those for control animals.

The findings of Issaeff were confirmed through exhaustive studies made by Pfeiffer (1895b) with the sera of goats which had been subcutaneously immunized for prolonged periods with increasing and finally with enormous doses of living cholera vibrios Even the most potent of these sera were found to exert " no true antitoxic effect " either against the toxin of chloroform-killed cholera vibrios or against that contained in broth cultures of

V cholerae which had been sterilized with toluene after an incubation for 20 days

It seemed at first glance in contrast to these observations that guinea pigs which had been injected intraperitoneally with a mixture of the toxic substances and large amounts of the immune serum tolerated toxin doses two to three times in excess of those lethal for the controls Pfeiffer found however that almost as high a tolerance for increased toxin doses could be produced if instead of immune serum the serum of normal goats was used for such tests He assumed, therefore that under the conditions of these experiments the immune as well as the normal serum exerted merely an unspecific action by hampering and retarding the absorption of the cholera toxins

A further interesting study of the problem presently under review was made by Pfeiffer & Friedberger (1908a) who used for this purpose El Tor sera put at their disposal by Kraus in comparison to a purely bactericidal cholera serum obtained through single intravenous injections of rabbits with minimal doses of *V cholerae* The El Tor toxin used to test these sera was obtained by (*a*) centrifuging the peritoneal exudate of guinea pigs which had succumbed to intraperitoneal infection with *V El Tor* and (*b*) adding to the supernatant small amounts of the bactericidal cholera serum so as to lyse the organisms which had not become sedimented.

In confirmation of the experiences of Kraus and Kraus & Russ, Pfeiffer & Friedberger found that the acute effects of the thermolabile exotoxin produced by *V El Tor* were neutralized by El Tor serum They stated however that

" in the case of the true toxins and antitoxins we find an exactly quantitative relationship the lethal dose is either neutralized or not. In the case of the mixtures of Tor exudates with Tor serum things are not so simple. Here one observes, even when manifold multiples of the protective minimal [serum] dose are used, a drop of the temperature [of the test guinea-pigs] down to 34 C, which may be accompanied by other signs of intoxication (most marked prostration) and which appears characteristically only 5-6 hours after injection of the mixture " [Trans.]

Pfeiffer & Friedberger concluded from these observations that the peritoneal exudates of El Tor infected guinea pigs contained two kinds of toxins—a neutralizable exotoxin and a second component, which represented the endotoxin liberated in the peritoneal cavity of the animals through disintegration of the organisms and which, as shown by a large series of carefully conducted experiments, was not neutralized by the El Tor sera. Pfeiffer & Friedberger further adduced evidence to show

" that in El Tor infection the antitoxic serum acted mainly through its bactericidal component, whereas the antitoxin contained in the serum according to our quantitatively conducted experiments was incapable of exerting a favourable influence on the course of the infection " [Trans.]

Preventive and curative tests made with the El Tor sera in cholera infected guinea pigs convinced the two workers that

"the El Tor antitoxin is by no means a universal antitoxin as is postulated by Kraus & Russ. It falls completely as far as the endotoxins of the cholera vibrios and also as far as the toxic substances are concerned which form in the body of cholera-infected guinea-pigs." [Trans.]

Entertaining no doubt that in the case of cholera as well as in that of El Tor infection the immune sera exerted only a bactericidal effect, Pfeiffer & Friedberger concluded that "so far the investigations of Kraus had furnished no proof for the existence of true antitoxins against the toxin of *V cholerae*"

The views of Pfeiffer & Friedberger were fully shared by Raskin (1909) who retesting Schurupow's serum found that this product, while exerting no anti-endotoxic action possessed a high bacteriolytic titre In accordance with these observations Raskin came to the conclusion that the curative action of Schurupow's serum in guinea pigs was not superior to that shown by purely bacteriolytic sera.

Pottevin (1913b) found that a classical strain of *V cholerae* which had been isolated in Constantinople possessed only a thermostable endotoxin which was neutralized neither by its homologous serum, produced through prolonged subcutaneous immunization of a donkey nor by the serum raised in an identical manner with one of the two El Tor strains examined at the same time The latter two strains possessed also an exotoxin which (*a*) was thermolabile (*b*) was endowed with haemolytic properties, (*c*) in contrast to the endotoxin of the cholera and El Tor vibrios was lethal for intravenously injected pigeons and (*d*) also in contrast to this endotoxin was neutralized within certain limits by either of the two above mentioned donkey sera.

These interesting observations, besides confirming the views held by Pfeiffer in regard to the toxin of *V cholerae* also speak in favour of an identity of the El Tor exotoxin with the "haemotoxin" (haemolysin) of these organisms The continued presence of endotoxic properties after the action of the exotoxin had been inhibited by heating, as demonstrated by Pottevin, was probably responsible for the belief of some workers that the *V El Tor* produced a soluble toxin distinct from its haemolysin.

Carrière & Tomarkin (1910) summarized the results of an exhaustive experimental study on the problem of cholera serotherapy made in Kolle's laboratory at Berne Switzerland as follows

"1) Bacteriolytic or purely bactericidal cholera sera obtained through a few intravenous injections of cholera vibrios possess the least curative effect, even when their lytic and agglutinating titres are high.

"2) The greatest curative effect in animal experiments is exerted by sera which have been produced through prolonged immunization with cholera bacteria, particularly if a mixture of the sera of different species of animals, immunized by various methods is used.

"3) Such sera contain considerable amounts of anti-endotoxins, but it is difficult to assess the anti-endotoxic properties in view of the comparatively low neutralizing power of the sera and the varying resistance of animals to the cholera toxins.

"4) The law of multiples is not valid for the anti-endotoxins of the cholera serum.

"5) The administration of comparatively large amounts of bactericidal serum is innocuous for animals with experimentally produced cholera peritonitis, provided that considerable amounts of anti-endotoxic substances are administered simultaneously

"6) Serum treatment of cholera patients is innocuous, if one uses instead of purely bactericidal sera, sera with some anti-endotoxin content In contrast to the widely accepted opinion that owing to the action of the bacteriolysins serum administration leads to the massive liberation of endotoxins, our sera as well as those manufactured in Russia caused absolutely no harm even if given to cholera patients in very large doses.

"7) To obtain therapeutic success, the administration of considerable amounts of serum is indispensable.

"8) It is essential to use for therapeutic purposes exclusively those sera which have been obtained through immunization of different animals prolonged as much as possible and of different animal species by subcutaneous and intravenous administration of living and killed cholera vibrios as well as of endotoxins and extracts of the latter" [Trans.]

Commenting upon the results of their studies, made mainly with the sera of goats, horses, and rabbits Carrière & Tomarkin found no reason to abandon the concept of the cardinal importance of the cholera endotoxins, but argued against the opinion often advocated that it was impossible to produce antitoxins against them. However while claiming to have proved the fallacy of such beliefs Carrière & Tomarkin admitted that the endotoxic substances at work

"probably do not represent the whole endotoxin of the bacterial cells in its original form and produce antibodies which do not completely correspond to the endogenous bacterial toxins. The incomplete efficacy of the sera might be due, therefore not to the fundamental impossibility to produce anti-endotoxic substances, but merely to the inadequacy of the methods available for immunization" [Trans.]

Kolle (1909a) in a preliminary communication on the work of Carrière & Tomarkin, expressed full agreement with the views of these two workers and maintained that

"prolonged immunization leads, perhaps more in some animal species than in others, to the formation not only of bacteriolysins and agglutinins but to some extent also of anti-endotoxins and, further of complement-fixing substances and bacteriotropins. Moreover this enumeration probably does not exhaust the substances which are involved in the treatment of cholera. It would be wrong, theoretically to dismember an immune serum and to conclude from such theoretical considerations upon its efficacy in animal experiments and in patients." [Trans.]

However while one must agree with Kolle that the mechanism of passive cholera immunity is of a more complex nature than is indicated by the rigid dicta of Pfeiffer and his school, and that anti-endotoxins probably play a role in it, one should not lose sight of the fact that all cholera sera produced thus far exhibited but feeble anti-endotoxic properties if any at all It is under these circumstances not surprising that, as

will be further discussed in Chapter 9 in spite of the optimistic opinions expressed by some of the early observers the inefficacy of cholera serotherapy is now generally admitted (see Hetsch 1928 and particularly Kraus 1929) In fact, apart from a solitary attempt made by Ghosh (1935 1936) the method of treating cholera patients with the aid of immune sera has not been used any more

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Chapter 5

BACTERIOPHAGE INVESTIGATIONS

Early Observations

In marked contrast to the copiousness of later records, during the period of about a decade following the discovery of bacteriophagy by Twort (1915) and by d Hérelle (1917) publications on the role of bacteriophages in cholera were rather scanty

D Hérelle himself (1922 see also d Hérelle Malone & Lahiri 1930) referring to his early observations in this respect, stated that

> " Amongst about one hundred cases of cholera studied in Indochina it was possible to observe but one following recovery In this last, in spite of daily examinations of stools, in but a single specimen taken at the beginning of convalescence has a bacteriophage active for the vibrio been found. This gave about 50 plaques when planted on agar In spite of many attempts it has been impossible to cultivate it by serial transfers. None of the fatal cases yielded a bacteriophage "

Continuing to work with cholera stools after the above mentioned observations had been made in 1920 d Hérelle (1923) was able regularly to demonstrate the presence of a lytic principle in the stools of cholera patients by

> (*a*) incubating broth dilutions or suspensions of the faeces for about 12 hours at 37°C (*b*) filtering the fluids first through kieselguhr and then through candles, and (*c*) after admixing agar-grown cholera vibrios to obtain a concentration of 100 million organisms per ml incubating the mixtures for 12 18 hours at 37°C, at the end of which period the vibrios were found to have been dissolved

The lytic principle began to become manifest in the cholera faeces 3-4 days after onset of the disease and continued to be present until the death of the patients and in the case of the single convalescent examined, up to the end of an observation period of about two weeks Similarly, in the case of one cholera vaccinated individual the lytic principle became manifest on the fourth day after the first inoculation and disappeared two weeks after a second dose of vaccine had been given 8 days after the first

D Hérelle felt convinced that the above-described phenomena were not due to bacteriophage action, because (1) in contrast to what was observed

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infected rabbits though not on one of the stock strains used for the immunization of his animals Noting these as well as further discrepant results obtained with other filtrates and/or cholera vibrios of different origin, Petrovanu maintained that these organisms were evidently apt to undergo variations, sometimes even rapid changes, in their behaviour to bacteriophages.

A valuable study of the susceptibility and the resistance of cholera vibrios to bacteriophage action was made by Flu (1924, 1925) As excellently summarized in the *Tropical Diseases Bulletin* (1926), the thesis of this worker was

"that strains resistant to bacteriophagy are, or may be, not only resistant, but also may by their growth, especially in bouillon, bring about a steady diminution of potency of any bacteriophage which may be present there would naturally be failure to carry on bacteriophage in subpassage, if the resistant strains got the upper hand and brought about the disappearance or inactivity of the bacteriophage.

"This idea of the author may further be set out as a number of propositions

"(1) A killed lysogenic strain in association with a young growing lyso-susceptible *strain in bouillon culture often furnishes a potent bacteriophage.*

"(2) Lysogenic strains cultured in bouillon often develop resistant strains with lengthening of the incubation time.

"(3) Strains which are resistant to bacteriophage bring about with their growth, disappearance of any bacteriophage present."

Flu pointed out that these considerations explained on the one hand why d'Hérelle, using too long an incubation period for his test specimens had usually failed to demonstrate the presence of cholera bacteriophage and made it clear on the other hand why Meissner who evidently worked with a specially lysogenic strain, obtained positive results even in tests with filtrates from the peritoneal exudate of normal guinea pigs.

Finding it legitimate for the reasons stated above to utilize again ten of the cholera strains which had given negative results in his 1923 bacteriophage tests, Flu adopted the following technique

"Of each of these strains the well-grown layers on four agar slants were suspended in 3 ml broth or normal saline and the suspension was mixed with sufficient anhydrous Na_2SO_4 for a solid mass to be formed. This was finely triturated in a mortar the powder was suspended in 100 ml broth, which was then heated for one hour at 58°C. The cooled broth was afterwards distributed into ten flasks, each of which contained 100 ml of broth. Each of these flasks was then seeded with one of the test cholera strains. One obtained thus ten test series, each consisting of ten flasks.

"After an incubation for two weeks a few ml of the contents of each flask were heated for 1 hour at 58°C and 0.1-ml quantities of these fluids were used for bacteriophage tests with each of the cholera strains under examination." [Trans.]

Tests carried out with the aid of the above-described technique showed on the one hand that only one of the ten strains examined was markedly lysogenic, i e capable of exerting a lytic action on phage susceptible

in the latter case, there was a strictly quantitative relation between the concentration of the filtrates and the amount of cholera vibrios per ml they were capable of dissolving, filtrates diluted to 50% lysing only 20-25 million organisms per ml and those diluted to 10% exerting no action even upon small inocula (2) serial transmission proved impossible and (3) the characteristic plaque formation remained absent when suspensions of cholera vibrios in active stool filtrates were spread on agar plates. D Hérelle was inclined, therefore to ascribe the observed vibriolysis to the action of a bacteriolytic (" diastatic ") ferment

It is interesting that afterwards a similar claim was made by Bernard & Guillerm (1933a) who stated that they had obtained by a method suitable for the extraction of diastases from bacteriophage free broth cultures of *V cholerae* a substance endowed with transmissible lytic power As these two workers formulated it in a second note (1933b) this substance had " the characters of an activator of the diastase which in normal cultures produces an autolysis of vibrios " However even if acceptable at face value, these observations cannot invalidate the now generally accepted, because fully confirmed belief in the virus nature of the bacteriophages.

D Hérelle s initial observations, which testified to the occurrence of true bacteriophagy in the case of cholera, found early confirmation through Jötten (1922) who in one instance demonstrated specific phage activity of a filtrate derived from a cholera stock strain Flu (1923) on the contrary obtained entirely negative results with the filtrates from 13 cholera strains which had been subcultivated monthly after they had been isolated seven years previously in Java.

Similarly Ciuca (1923) found the filtrates obtained from the faeces of five cholera patients (four of whom recovered) active only against dysentery bacilli and *E coli* but not against the 17 *V cholerae* strains tested. However as stated by Petrovanu (1924a) Ciuca and he had afterwards been able to demonstrate a weak lytic action of cholera stool filtrates on heterologous strains of *V cholerae* not directly isolated from patients

Meissner (1924) making " incomplete " Pfeiffer tests by intraperitoneally injecting guinea pigs with lethal doses of *V cholerae* and small amounts of cholera immune serum, found that filtrates of the peritoneal exudate of such animals displayed a typical bacteriophage activity which was serially transmissible Working obviously with phage-contaminated materials she obtained identical results with the peritoneal exudates of guinea pigs intraperitoneally injected only with nutrient broth.

In contrast to the findings of Meissner Petrovanu (1924a) was unable to demonstrate a lytic action of the peritoneal exudate of rabbits injected intraperitoneally with lethal or non lethal doses of *V cholerae* However Petrovanu (1924b) found that filtrates from the washings of ground up small intestines of cholera immunized rabbits exerted a typical bacteriophage action on the organisms freshly isolated from the heart blood of cholera

infected rabbits though not on one of the stock strains used for the immunization of his animals Noting these as well as further discrepant results obtained with other filtrates and/or cholera vibrios of different origin, Petrovanu maintained that these organisms were evidently apt to undergo variations sometimes even rapid changes in their behaviour to bacteriophages.

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strains, and on the other hand that not all of the remaining nine strains were susceptible to lysis. In fact, as established by Flu,

"only three [of these] strains were found to give lysis under all circumstances and to lead to plaque formation on the plates and slants, and only these out of 20 strains of my stock could be used for subcultivation of the bacteriophage." [Trans.]

The strongly lysogenic strain was not only lysoresistant against the bacteriophage obtained from it, but also capable of inhibiting bacteriophage action in fluid media and to lead after repeated subcultivation in such media to a complete disappearance of the bacteriophage

Nobechi (1926a) while unable to demonstrate the presence of cholera bacteriophages with the aid of the techniques of Jötten Meissner or Petrovanu found the method recommended by Flu effective However Nobechi stated that he had obtained at least equally good results by simply filtering the bacteriophage-containing broth cultures

Making cross bacteriophage tests with the 18 *V cholerae* strains at his disposal and their broth culture filtrates, Nobechi found 3 of these strains to be bacteriophage resistant and lysogenic, while a second group of 6 strains proved to be resistant but non lysogenic, and a third lysosensitive and non lysogenic. It was noted that the clarification of broth cultures of lysosensitive strains under bacteriophage influence began to become manifest after 3 hours and became maximal after 4-5 hours It was essential, therefore, to observe the tubes continuously from the beginning of the tests, because in the case of delayed examination rapidly occurring secondary growth could mask the presence of bacteriophage Nobechi also advised making subcultivations during the phase of maximal lysis

Studying the mutations of *V cholerae* under the influence of bacteriophage Nobechi (1926b) found that these were not of a permanent character reversion to type took place after some passages in bacteriophage-containing broth media, and the strains in question became at the same time resistant to bacteriophage action, non lysogenic, and also spontaneously agglutinable However spontaneous agglutination was no longer observable when such strains were transplanted on agar

Nobechi further found that intraperitoneal injection of guinea-pigs with 0.3-0.5 ml of bacteriophage cultures protected the animals against intraperitoneal challenge with 3-10 MLD of *V cholerae* the phenomena of Pfeiffer s reaction becoming manifest in such animals Bacteriophage administration by the oral, subcutaneous or intravenous route as a rule did not protect the animals against intraperitoneal challenge The filtrates of either lysogenic or non-lysogenic cultures also showed only slight protective power Intravenous injections of bacteriophage did not effect a disappearance of the organisms from the gall-bladder of experimental animals which had become carriers of *V cholerae*

In his work *The bacteriophage and its behaviour* d Hérelle (1926) besides referring to his own previous experiences and to those of some of

the other workers mentioned above recorded only one observation he had made more recently

"In 1924 during the return of the pilgrims from Mecca, while they were quarantined at the lazaretto of Tor I isolated from the stools of one of these pilgrims (although he showed none of the symptoms of cholera) a vibrio which agglutinated with an anti cholera serum to its titre While held in the lazaretto the stools of this pilgrim were examined every 48 hours. After a time the stool revealed a non agglutinating vibrio and after a further 48 hours all the vibrios had disappeared

"The vibrio susceptible to agglutination was bacteriophaged perfectly by the bacteriophage isolated by Flu while the vibrio which was inagglutinable, isolated 48 hours later was refractory At this time the intestinal contents contained a bacteriophage having a virulence for the agglutinable vibrio"

Interesting as this observation is the information supplied by d Hérelle is too scanty to show whether the "inagglutinable" vibrio found after the disappearance of the specifically agglutinable organisms was a rough cholera vibrio It is quite possible therefore that the appearance of not specifically agglutinating vibrios in the stools of the pilgrim was of an accidental nature

To complete the present record reference has to be made again to the observation of van Loghem (1926) already quoted in the preceding chapter that bacteriophage action hastened the liberation of the endo haemolysin of *V cholerae*

Later Investigations

A new epoch in the history of the subject presently under review began in the year 1927 when large-scale investigations on cholera bacteriophagy were started in India under the auspices of the Indian Research Fund Association by d Hérelle and co-workers (see d Hérelle & Malone 1927) and also by Morison (see Morison & Vardon, 1929) While as will be described below Morison and his colleagues continued to make important contributions to the knowledge on cholera bacteriophages and their role in the prevention and cure of the disease, d Hérelle s work was taken over in 1928 by Asheshov (see Asheshov et al 1930) who published final reports on the experiences made by him and his staff in 1933 (see Asheshov et al. 1933)

The main results obtained by these and some other investigators working in India or elsewhere as far as they fall under the scope of the present disquisition may be discussed under the following headings

Types of cholera bacteriophages

In their final report (Part II) Asheshov et al (1933b) thus described the properties of the three cholera bacteriophages they had detected early

in their work (Asheshov et al 1930) and of two additional phages found by Pasricha De Monte & Gupta (1932a)

"ChφA (i.e. cholera phage A) is a quick acting bacteriophage the best race of this can produce complete lysis of vibrios in less than two hours Its generation period is approximately between 45 minutes and 1 hour 15 minutes.

"The lysis is never permanent and is quickly followed by abundant secondary growth resistant to all bacteriophages of the type A. It attacks only the smooth elements of the culture, not touching the rough. The members of this group vary widely in activity range virulence and stability The virulence can be exalted, particularly if bacteriophage is freshly isolated. But the great majority of the freshly isolated ChφA are very unstable. They die out within a very short period—sometimes within a few days—unless they are adapted to laboratory conditions by frequent transfers. The range of virulence of some of the races of this type is often restricted to a small number of strains of vibrios, but they are comparatively easily adapted to act on the other strains of smooth cholera vibrios. They do not attack the non-agglutinable vibrios even if the latter are smooth.

"ChφB. The generation period of this bacteriophage on a smooth-rough culture is usually between 1 hour 15 minutes and 1 hour 45 minutes and the lytic action of even the most active of this type is considerably slower than that of ChφA. The lysis is seldom produced in less than three hours. The lysis is also not permanent and is followed by the secondary growth which appears later than with the type A. On the other hand, ChφB is considerably more stable than ChφA. The range of virulence is very wide ChφB acts on both smooth and rough elements attacking also some of the non-agglutinable vibrios.

"ChφC is a slowly growing bacteriophage, with generation period of 2 hours to 2 hours 30 minutes. It produces appreciable lysis only with rough cultures and even then seldom complete and followed by the usual secondary growth ChφC acts on the culture better on the agar surface than in the broth. The range of activity of this type is very wide we have not yet met a strain of cholera vibrios which is not acted upon by our ChφC. It attacks also many non-agglutinable vibrios.

"ChφD (Pasricha). The generation period of this bacteriophage is 1 hour 20 minutes to 1 hour 30 minutes on a smooth and 1 hour 30 minutes on a rough strain. The lytic action is slower than that of ChφB but quicker than that of ChφC it gives an incomplete lysis in about five hours The range of activity and stability of this bacteriophage have not yet been sufficiently studied.[1]

"ChφE (Pasricha) A very slow acting bacteriophage with generation period on rough culture of 1 hour 40 minutes to 1 hour 50 minutes seems to act only on rough elements. The lytic action is very slow but is more pronounced than with ChφC the lysis of rough culture is more complete than with ChφC and the secondary growth appears with difficulty"

Important additional information on these choleraphages, particularly the first three types was supplied by Morison (1932) Rao (1932) and White (1936b 1937)

Morison (1932) stated that cholera vibrios, if made resistant to type A choleraphages, became as a rule though not invariably rough in character and also drew attention to the fact that, though the strains isolated from patients were usually smooth in character batches found at the end of one Calcutta outbreak had been mostly rough and at the same time

[1] According to Pasricha, De Monte & Gupta (1932a), the cholera D phage had a greater range of activity than any other choleraphage

resistant to cholera A phages However while admitting that these findings were in accord with those made by d Hérelle (1926) Morison stated that he was "not yet in a position to comment on the claim that resistant strains are avirulent"

In Morison s experience cholera strains which had become rough and resistant to type A choleraphages became apparently changed into smooth strains if they were made resistant also to type B or type C phages Thus, as this worker put it, the type A choleraphage seemed "to be a factor in producing rough and B and C factors in producing smooth strains"

Rao (1932) studying the relationship between the action of cholera phages and the reaction of the media used for such work, reached the conclusion that

"Within the optimum limits lysis of the type A cholera bacteriophage is enhanced by increasing acidity and that by type B by increasing alkalinity"

Rao maintained therefore with much reason that differences in the reaction of the media used might exert an influence upon varying results obtained in cholera bacteriophage work.

White (1936b) stressed that the choleraphage A exerted a lytic action solely upon classical cholera and El Tor strains. Positive reactions obtained with such strains were, therefore of diagnostic importance, but it deserved attention that some of the type A phages failed to attack Inaba type strains of *V cholerae* Another important fact was that alone among the cholera phages known to White the A type phages lysed only the S form of the vibrios they were capable of attacking, but not the R races isolated from the smooth strains without the intervention of bacteriophage In analogy with this observation the secondary growths resulting from the action of cholera A phages were "essentially if not always entirely rough"

Further studying two cholera A phages he had obtained from Morison and from Asheshov respectively White (1937) found that both these phages attacked all Ogawa type cholera strains regardless of their geographical origin Indian Inaba strains were attacked by Asheshov s A phage but not by that of Morison. Neither of these phages could be cultivated either in peptone water or on agar on the Chinese or Japanese cholera strains at White s disposal unless the activity of the phages was enhanced by the addition of egg white ("lysozyme") to the cultures in question.

In order to turn attention to the discovery of choleraphages additional to those enumerated above it has first to be stated that Pasricha De Monte & Gupta (1932b) finding a bacteriophage, which had been grown originally on a cholera like vibrio capable of attacking the secondary growths produced through the action of the hitherto known choleraphages, classed this new type as choleraphage "F" Further as summarized by Pasricha and colleagues (1936) (1) Morison (1933 see also a preliminary remark made by

this worker in 1932) reported on the isolation of three new choleraphage types "G" "H" and "J" (2) Pasricha (1933) found a "K" type, active for rough vibrios only (White 1937) (3) an "L" type was reported in 1935 by Anderson and (4) an "M" phage had been recently isolated from a vibrio strain not agglutinable with cholera immune serum which had been cultivated from the faeces of a cholera patient. As noted by Pasricha and colleagues (1936) this new phage, while slow in action, had a wide range of activity attacking some cholera like vibrios as well as most cholera strains

Commenting upon these observations, the same authors (1936) emphasized that among the choleraphages known thus far the A phage alone was restricted in action to vibrios agglutinable with cholera immune serum and acted, moreover solely on organisms possessing a considerable degree of smoothness The choleraphage A was also immunologically in a class of its own, because sera raised with it inhibited only the action of their homologous phage whereas serological relationships existed in the case of the other choleraphages

As stated by White (1937) he proposed to continue work with the L choleraphage However he lost the strain which had been isolated originally by Anderson (1935) from Calcutta sewage and a second strain labelled phage L received afterwards was found incapable of lysing the cholera strain attacked by the original L phage White considered it advisable therefore, to call the second strain at his disposal the "LL" phage thus indicating that it was probably distinct from the original type Summarizing his experiences with this second strain White stated that the LL phage

"is possibly the most frequently occurring of Indian cholera phages. The majority at least of cultures of *V cholerae* from Indian sources are LL-lysogenic. On the other hand those Chinese and Japanese strains of *V cholerae* which have been so far examined have been found LL-free and LL-sensitive While all *V cholerae* examined have been found either infected with or sensitive to LL, neither of these conditions has yet been detected in El Tor and other vibrios."

While as will be seen below the LL phage does not quite show the limitations in geographical distribution postulated by White the observations of this worker on the restriction of the distribution of this phage to the classical *V cholerae* are of great interest.

Another interesting fact established by White was that the usually feeble lytic action of the LL phage could be enhanced by the addition of "lysozyme" (i e egg white in a 1 25 concentration) to the culture media. As already alluded to White also found that two A type cholera phages, which failed to multiply on Far Eastern Inaba strains in spite of their specific affinity for the polysaccharides of these strains attacked the organisms in question vigorously in the presence of the egg white lysozyme Where no such affinity existed, the lysozyme failed to promote

bacteriophagy In White's opinion this observation supported the view "that the combining property of bacteriophage is to be clearly distinguished from its lytic activities"

In apparent agreement with this view White found that though no signs of bacteriophage action became manifest when *rough* LL free races of *V cholerae* were exposed on agar plates to the action of this phage it could multiply indefinitely upon the R cultures which, like the smooth growths became lysogenic He added that

"Cholera phage LL is fixed and inactivated by the polysaccharides of S,R and ρ *V cholerae* and probably has a specific affinity for the substance which I have termed C γ (White, 1936[a])"

Continuing work with the LL phage Pasricha Lahiri & De Monte (1941) established that (*a*) the secondary cultures obtained after the action of this phage were lysable by the 12 other types of choleraphages (A→M) while (*b*) the LL phage acted reciprocally on the secondary cultures resulting from the action of each of the other types of choleraphages. White's assumption that the LL phage was a new type being thus confirmed, Pasricha and co-authors proposed for it the name of choleraphage "N"

Like choleraphage A the N type exerted no action on cholera-like vibrios. Evidence of its presence could be found in only 3 out of 115 recently isolated cholera strains but it is noteworthy that these had all been obtained within a short time from one and the same locality Positive results were obtained with all Indian stock cholera cultures and, in contrast to White's experiences, also with some strains from Hong Kong, three of which yielded N phage while a fourth proved resistant and a fifth sensitive to the action of this phage In confirmation of White's observations, Pasricha and colleagues found egg white lysozyme an excellent means of propagating the N phage but they obtained satisfactory results also with solid or semi-solid agar

In their article describing the D and E types of choleraphages, Pasricha De Monte & Gupta (1932a) also stated that they had isolated from the water of the Hooghly river a "W" phage found to be capable of lysing a number of cholera like strains. Though the latter had proved to be resistant to the action of choleraphages A→C, the secondary growths appearing after the action of W phages were found to be lysable by cholera phages

Subsequently Pasricha and colleagues (1932b) referred to the isolation of 8 (or to judge from their protocols rather 9) strains of *vibriophages* found to be active against cholera like strains which were not lysed by cholera phages. After repeated passages on their respective vibrios, 5 of these phages acquired the property of lysing cholera vibrios However as stated by the same authors in 1936 such vibriophages, "if active on agglutinable (i e cholera) vibrios do not give the reciprocal cross test"

To judge from a short remark made in 1934 by Russell (1935) up to then over 30 different races of vibriophages had been isolated in India.

Pasricha De Monte & Gupta (1931a) studying the seasonal incidence of choleraphages in Calcutta, found that

"(a) Cholera 'phages in Nature vary with the incidence of the disease. It is rare to isolate cholera phages from water during the non-cholera season.

"(b) The mortality rate which is high at the beginning of the cholera season, falls rapidly when cholera phages have become widely distributed in Nature. The spread of bacteriophages thus apparently plays a very important role in the lowered mortality and in bringing an epidemic to a close

"(c) Cholera phages in Nature are of the quick-acting type A, and evidence is presented suggesting that the types B and C die out in Nature."

These conclusions are in accord with the postulations of d Hérelle and his co-workers to which reference will be made later They stand, however in a curious contrast to the contention of Morison (1935) that, unlike other phages types cholera A phages though frequently met with in the patients "may have little effect on the infectivity or virulence" of *V cholerae*

Fixation and inhibition of choleraphages

White (1936b) pointed out that observations by several workers, made with bacterial species other than the vibrios, had demonstrated the power of specific extracts separated from the organisms in question to fix and inactivate particular bacteriophages. He made therefore corresponding tests with substances extracted from vibrios and choleraphage types A→J White was thus able to show that

(1) the A-type phage was selectively inactivated by the smooth specific polysaccharide of cholera and El Tor vibrios

(2) the polysaccharides derived from cholera-like vibrios as well as those from R and ρ races of *V cholerae* exerted no inhibiting action on the A-type choleraphage

(3) "lipoid" constituents of the cholera vibrios, obtained by alcohol extraction, while in properly conducted tests not interfering with the action of choleraphage A (and also of the type D), specifically inhibited the phage types C, E, G and H, while the B-type phage was not invariably inhibited by these substances.

White concluded from these observations that, regardless of whether the polysaccharides or the lipoids specifically bound the phages, "in both cases the resistance of the secondary culture is probably due to its loss of susceptible substance rather than to any positive modification or immunity"

Pandit, Maitra & Datta Roy (1936) who also made an early study of the problem presently under review used the following technique to prepare extracts of cholera and other vibrios

"The organisms were grown in Roux's flasks for 48 hours. The growth from each flask was washed and emulsified in 10 c.c. distilled water The emulsions

were placed in a water bath at 55 C for 72 hours. They were then diluted by adding twice the amount of distilled water and filtered through a Seitz filter "

Applying this technique Pandit and co-workers tested the inhibitions produced by the extracts of 17 strains of cholera El Tor and cholera like vibrios on choleraphages A →K It was found that the phage typ s C and G were not inhibited by any of the extracts tested and that, on the other hand, the extracts of two cholera like vibrio strains as well as that of the single available El Tor strain did not inhibit the action of any of the above mentioned bacteriophage types.

No definite correlation appeared to exist between phage type inhibition and resistance of the strains to the phages in question but multiple type resistance was found to be associated with a diminution in the number of types inhibited A parallelism was found to exist between the types of phage inhibition produced by the extracts of the strains and the polysaccha ride content of the organisms concerned according to the classification of Linton and his co-workers (see Chapter 3)

Continuing the above-described work, Maitra (1939) combined phage inhibition tests (types A →L) with precipitin tests, made with sera raised against strains falling into different groups according to the type of their phage inhibition and polysaccharide content. It was found that according to the results obtained by such combined tests the vibrios could be divided into two groups, namely one consisting of typical smooth cholera vibrios and El Tor vibrios in the strict sense and a heterogeneous group comprising atypical cholera vibrios, particularly rough strains, and cholera like vibrios Maitra concluded in contrast to the statements of Pandit and colleagues that

" Both the inhibition and precipitin reaction appear to depend on a common factor which is related to the complex polysaccharide receptor of the cholera vibrio but bears no direct relation to the type or quality of polysaccharide by chemical analysis as found by Linton."

As stated in a preliminary note by Doorenbos & Cossery (1950) a serum raised in rabbits against cholera B phage besides possessing weak agglutinating properties (titre 1 300) inhibited in dilutions of 1/1000 after an incubation for 4 hours the activity of this phage

Action of choleraphages on El Tor vibrios

Though repeated references are made elsewhere in the present chapter to the behaviour of El Tor vibrios under the action of choleraphages, it is necessary to deal separately with the observations made in this respect by Jadin (1936)

Making comparative tests with a cholera bacteriophage Jadin found it far more active for classical cholera than for El Tor vibrios the former organisms were completely lysed when the phage was used at a dilution

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"In a word if a powerful bacteriophage is brought into contact with cholera vibrios the latter are parasitized and then rapidly and finally destroyed. If the bacteriophage which attacks them is less potent a certain number of vibrios resist but *contract a chronic disease* transmissible to their descendants, which has the effect of modifying, more or less profoundly their characters."

While the fargoing conclusions which d Hérelle and his colleagues drew from the above and allied observations will receive attention later the following records of other investigators deserve consideration at the present juncture

Finkelstein (1931) drew attention to unpublished observations according to which the phage resistant growths appearing after the action of a choleraphage fell into two types in regard to their agglutinability, one showing a granular and the other a large flake agglutination This dissociation which appeared to be stable in character, was accompanied by a loss of the motility of the organisms

Chen (1932) testing two cholera strains with the aid of a cholera dysentery phage noted the successive appearance of three types of variant colonies, two of which showed signs suggestive of roughness, producing a granular growth in broth subcultures and showing spontaneous agglutination in saline suspensions Motility was found to have disappeared in the case of one of these variants and also in that of the third, apparently smooth, variant All three variants reverted to type after more or less prolonged subcultivation more rapidly after animal passage While the variants showed no marked changes in agglutinability they seemed to be less virulent for hamsters than the parent strain

These observations were supplemented by valuable findings made by Chen (1933) with 195 stool specimens of 21 cholera patients 18 of whom could be studied throughout their convalescence Chen found that the cholera colonies thus isolated

"were divisible into 3 distinct types according to their smooth and rough reaction, susceptibility to cholera phage and agglutinability with high titered serum. Smooth type lysible colonies by cholera phage with full titer agglutination was always isolated in abundance during early acute stage of the disease when cholera-phage had not yet appeared

"As soon as cholera-phage appeared in the stool and the patient was convalescent, smooth type colonies became scanty or entirely absent, and intermediate and rough resistant variants of comparatively low titer agglutination (1 160-1 640) took their place Motility of the rough variants was also sometimes impaired and non-motile colonies of rough cholera vibrio were isolated in 4 cases. The motility however could be restored on subsequent transfers in laboratory media."

In the case of five of the above mentioned 18 patients absolutely "non agglutinating" cholera-like vibrios appeared, which proved to be rough in tests with Millon s reagent and choleraphage resistant However cross agglutination and absorption tests with sera raised against (*a*) smooth cholera vibrios (*b*) slightly agglutinable rough variants, and (*c*) four of

of 10^{-8}, and after joint subcultivation for several months even at a dilution of 10^{-10} The El Tor vibrios though slightly lysed by 10^{-5} bacteriophage dilutions, were completely lysed only by 10^{-3} dilutions. Subcultivation of the phage on cholera vibrios did not increase its lytic power for *V El Tor* subcultivation of the phage on the latter organisms did not abate its potency for *V cholerae*

Heating of the bacteriophage dilutions for half an hour at 64°C did not deteriorate their lytic action for cholera vibrios but such heated dilutions produced no lysis of El Tor vibrios However it was possible to restore the activity of such phage dilutions for the *V El Tor* by repeatedly exposing them to a temperature of 75 C.

A serum raised with a bacteriophage active for cholera vibrios but inactive for *V El Tor* was found to inhibit the action of unheated choleraphages on the latter organisms, but only to diminish the phage activity against *V cholerae* The serum completely inhibited the action of choleraphages heated to 65 C.

It was further established that repeated subcultivation of an El Tor strain contaminated with a 10^{-6} dilution of bacteriophage rendered the latter capable of lysing El Tor vibrios, apparently because in the course of subcultivation a multiplication of the phages had taken place

Jadin concluded from these observations that the different behaviour of the choleraphage towards cholera and El Tor vibrios respectively was the result of differences in the phage sensitivity of the two organisms and not of the separate presence of an anti-cholera and an anti El Tor factor in the phage

Bacteriophage-produced vibrio variations

Reporting on their studies in India, d Hérelle Malone & Lahiri (1930 see also d Hérelle, 1930) stated that under the influence of bacteriophages the *vibrios underwent profound changes the motility of the organisms was apt* to become eventually lost their morphology became changed, bacillary and ultimately coccal forms making their appearance the reactions produced by the vibrios in carbohydrate-containing media became modified, and the property of reducing nitrates became lost. More important still bacteriophage action led to a loss of the specific agglutinability with cholera immune sera and also to a loss of virulence

Commenting upon their findings in this respect with the faeces of cholera patients, d Hérelle, Malone & Lahiri stated it to be " beyond dispute " that these changes in the properties of the organisms which had been observed as well under the action of bacteriophages *in vitro* " constitute real mutations of the typical cholera vibrio " They maintained in this connexion that

" When the loss or the modification of a character *is the result of a mutation*, this character apparently cannot be recovered. The mutation appears to be final and the return to the original form impossible, in contra-distinction to what is produced when the loss of a character is simply the result of disuse

"In a word, if a powerful bacteriophage is brought into contact with cholera vibrios the latter are parasitized and then rapidly and finally destroyed. If the bacteriophage which attacks them is less potent a certain number of vibrios resist but *contract a chronic disease* transmissible to their descendants, which has the effect of modifying, more or less profoundly their characters."

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the cholera like vibrios respectively showed a full antigenic independency of the latter organisms not only as far as the smooth cholera vibrios but also as far as the rough variants of the latter were concerned It was in accord with these observations that daily transfers in alkaline peptone water repeated for three months as well as animal passages, while producing a tendency to better agglutinability with cholera immune sera in the case of the rough variants yielded no such results in the case of the cholera like strains

An interesting study of the antigenic characters of three types of secondary cultures obtained with A, B and C choleraphages respectively was made by Pandit & Rao (1932) who thus summarized their results

> "The main change was in respect of presence of somatic antigen. This antigen was considerably diminished in the case of secondary A but it persisted in varying amounts with secondaries B and C. The above gradation was noted in the case of flagellar antigen also The power to provoke normal agglutinins was found to be proportional to the amount of somatic antigen present."

It is important to note that the rough variants of *V cholerae* produced independently of any phage action " showed more or less the same antigenic structure as was noted with A phage type secondary cultures " The variants appearing after action of choleraphages B or C could not be considered as truly rough in character because, as noted above they continued to contain some of the smooth somatic antigen

As already referred to in Chapter 4 Morison (1932) stated that he had been unable to make cholera vibrios specifically inagglutinable by growing them in the presence of bacteriophages he added that, when working under conditions that precluded contamination he and his colleagues had also been unable to transform true cholera into cholera like vibrios by other means

These observations were confirmed by Vardon (1940) who stated that the secondary growths, which appeared after the individual action of the 11 types of choleraphages known in 1935 on a typical *V cholerae* strain, were agglutinated to over 50% of the titre by an H + O serum raised against this parent strain. The sugar reactions of the secondary growths were also identical with those shown by the original culture

However Morison reported in 1935 that using combinations of the choleraphages instead of single types he and his co-workers had been able to effect

> " changes in the morphology the colonies on agar the growth in broth, the salt stability the agglutinability and the ability to ferment sugars which varied with the combinations of types of bacteriophage and the period of action. These changes ranged from slight to so profound that the bacteria resulting therefrom are quite unrecognizable as vibrios."

Some details on these and on analogous observations made during the following years with the aid of O-agglutinating sera raised against cholera and phage produced variant strains were furnished by Vardon (1940)

Though he found *in vitro* some changes in the serological behaviour of such variants, it is noteworthy that according to a statement made by Anderson (1937) the presence or absence of phages infecting the vibrios in nature did not appear to influence their agglutinability

Careful studies on the changes 16 typical non haemolytic and more or less phage-sensitive Indochinese cholera strains were apt to undergo under the influence of cholera bacteriophage were made by Bernard & Liang (1933) and by Bernard Raynal & Liang (1933) As stated by Bernard & Liang, the secondary cultures obtained from these 16 strains after the action of a choleraphage from Assam showed the following properties

(*a*) while no change in agglutinability was observable in four instances six of the variant strains showed an impairment and six a total loss of their agglutinability with the three cholera-immune sera used

(*b*) the proteolytic properties of the variant strains were not altered in six instances, whereas the others liquefied gelatin and coagulated serum with some delay and to a lesser degree, or were even altogether unable to liquefy such media.

As shown in the case of one of the strains, daily subcultivation of the phage produced variants on gelatin-agar led to a gradual restoration of the properties of the parent strain, which became complete after the seventh passage If such a " purified " strain was again contaminated with bacteriophage, the cycle of modifications was started once more Bernard & Liang maintained in this connexion that

" the isolation from the seven first passage cultures on gelatin-agar of colonies which had preserved an absolutely normal form shows that pure and modified colonies coexist in the same growths. In the course of successive passages, the pure colonies develop more rapidly than the contaminated colonies and restore, after a minimum of seven passages, the original culture. There can be no doubt that, depending upon the character of the original culture and the activity of the bacteriophage used, marked differences ought to exist in the number of passages necessary for the reconstitution of the growths to their original state." [Trans.]

Bernard, Raynal & Liang (1933) supplemented these observations by stating that the cholera red reaction positive in the case of all 16 original strains was more marked in that of six of the secondary growths appearing after bacteriophage action and weaker than normal four times The findings made by these workers regarding the behaviour of their strains in blood containing media will receive attention in a later part of this chapter

In order to assess the alterations of the properties of cholera vibrios under the influence of bacteriophage, Dambovіceanu Combiesco & Soru (1934) used six strains two of which were of a smooth character while two were rough and two were either in a transitory stage or consisting of a mixture of smooth and rough elements. Bacteriophage action produced no alteration of the specific agglutinability of all these strains and also did not alter the physico-chemical properties of the two rough strains However the variant growths developing after bacteriophage action in the case of the

the cholera like vibrios respectively showed a full antigenic independency of the latter organisms not only as far as the smooth cholera vibrios, but also as far as the rough variants of the latter were concerned. It was in accord with these observations that daily transfers in alkaline peptone water repeated for three months as well as animal passages, while producing a tendency to better agglutinability with cholera immune sera in the case of the rough variants, yielded no such results in the case of the cholera like strains

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As found by this worker bacteriophage action led also to other modifications of the properties shown by the parent strains while the colonies of the latter were endowed invariably with marked haemodigestive power after bacteriophage action this property became less conspicuous or was even no more apparent Further phage-contaminated strains showed a less abundant sometimes a granular growth in peptone water The cholera-red reaction, though markedly varying, was as a rule more strongly positive in the case of the phage-contaminated strains than in that of the parent strains The degree of gelatin-liquefaction produced by the phage-contaminated strains showed more marked variations than was the case with the parent strains sometimes the phage-contaminated growths failed to liquefy gelatin.

Testing 35 of his strains with two different cholera-immune sera, Doorenbos found that in the case of the parent strains agglutination took place both in the form of flakes and of granules Several of the lysogenic growths, on the contrary showed merely agglutination in the form of granules, which sometimes were hardly visible with the naked eye. One of the two sera, which had a low titre, sometimes failed altogether to agglutinate colonies of the lysogenic strains.

The general conclusion which Doorenbos reached on account of these observations was that

" it is possible (namely through the intervention of d'Hérelle s bacteriophage) to transform *in vitro* the agglutinable vibrio into a non-agglutinable vibrio a non-haemolytic vibrio into a haemolytic vibrio, a vibrio giving a feeble indol reaction into one giving a strong reaction to isolate from one and the same strain agglutinable and inagglutinable vibrios, haemolytic and non-haemolytic vibrios " [Trans.]

While most of these postulations of Doorenbos are acceptable because they are in agreement with corresponding observations made by other workers, exception must be taken to the claim that he had succeeded in transmuting cholera into El Tor vibrios For all that he was able to achieve in this direction was to produce quite unstable haemolytic variants of *V cholerae* whereas actually as has been shown in the third chapter the reactions given by cholera and El Tor vibrios respectively in properly conducted haemolysis tests are characterized by a great stability There can be no doubt, however that, as had been shown previously by van Loghem (1926) and was confirmed by the observations of Doorenbos as well as by the findings of Bernard Raynal & Liang (1933) and Scholtens (1935) recorded below the action of choleraphages is apt to exert an activating influence on the latent haemolytic properties of *V cholerae*

The experience in this respect, of Bernard and co-authors (1933) with 16 cholera strains may thus be tabulated

Reactions	*Parent strains*	*Secondary growths obtained after phage action* for 6 hours	for 24 hours
Haemagglutination (sheep erythrocytes)	uniformly positive	identical, 10 times stronger 5 times weaker once	identical 11 times stronger 4 times weaker once
Haemodigestion (rabbit-blood agar)	uniformly positive	identical, 9 times weaker 7 times	identical, 7 times weaker 9 times
Haemolysis (saline suspensions of sheep erythrocytes)	totally negative even after 48 hours	4 times positive after 24 hours, 9 times inconstant results	

smooth and transitory strains showed changes in their agglutinability with trypaflavin and in the zones of their acid agglutination as well as in the speed of their cataphoresis, exhibiting in these respects in contrast to the original growths the reactions characteristic of R strains

Damboviceanu & Soru (1934) studying the content in residual antigen of three smooth two transitory and one rough cholera strains before and after bacteriophage action, found that

(*a*) residual antigen, though abundant in the extracts of the smooth parent strains was no more demonstrable in the growths which had recently acquired rough properties under bacteriophage action

(*b*) however strains which already exhibited rough features before bacteriophage action yielded after as well as before this action extracts as rich in residual antigen as the smooth strains or even showed a higher content in residual antigen.

A study of the rough variation of *V cholerae* and its relation to resistance to cholera A phage by Yang & White (1934) led to the following conclusions of importance for the subject presently under review

"(a) According to the condition of the ultrapure culture of *V cholerae* exposed to A type choleraphage the resistant growth is predominantly smooth, intermediate or rough in serological character

"(b) Extreme rough variants isolated from ultrapure cultures without the help of A choleraphage are identical with those obtained by its use and seem to be invariably resistant to this agent

"(c) Attempts to isolate from ultrapure cultures, by methods of simple selection, variants resistant to A type phage have met with a single but apparently significant success. On the whole we are inclined to believe that resistance to A phage is not a modification induced by phage action but that resistant elements are present in the ultrapure culture and survive lysis."

As recorded by White (1937a) the action of the LL choleraphage resulted

"in a very variable and usually trivial tendency towards roughness in the surviving growth, which may lead to its behaviour as a mixed SR antigen, to some reaction with R agglutinin and even in certain cases to somewhat increased susceptibility to the precipitating action of NaCl"

The observations made in regard to the problem presently under review by Doorenbos (1932) and Scholtens (1935) as well as part of those by Bernard, Raynal & Liang (1933) deserve separate consideration because these workers paid particular attention to the modifications produced by the action of choleraphages in the behaviour of the vibrios in blood-containing media

Doorenbos (1932) reported in this connexion that he had worked with 70 cholera strains which were originally lysosensitive and incapable of lysing goat erythrocytes. After having been artificially contaminated with bacteriophage, 14 of these strains became strongly haemolytic. Though finding that these haemolytic variants were not stable, their subcultivation leading to the appearance of non-haemolytic as well as of haemolytic colonies, Doorenbos felt entitled nevertheless to claim an *in vitro* transformation of cholera into El Tor vibrios.

cholera vibrios at high titre As also noted Pasricha and his colleagues even though finding this acquired agglutinability difficult to maintain, ascribed great significance to their observations feeling convinced that a large proportion of the "non agglutinable" vibrios present in cholera affected localities were mutant forms of *V cholerae* apt to play a considerable role in the causation of the disease However discussing the origin of the phage resistant and specifically agglutinating vibrios obtained after bacteriophage action on cholera like vibrios, these workers admitted the difficulty of refuting the criticism that the apparently transmuted organisms might have been pre-existent in "a very small proportion" in the original growths. There is no doubt in the mind of the present writer that this interpretation of their findings and of some analogous results afterwards recorded by Vardon (1940) is by far the most likely one

In their 1932 paper Pasricha De Monte & Gupta referred to one "non agglutinating" strain isolated from the faeces of a patient who showed clinical features of cholera stating that (*a*) the vibrios in question were lysable by a bacteriophage isolated from water (*b*) the secondary growths developing after the action of this "vibriophage" were lysable by cholera phages B C, and D and (*c*) these secondary growths were agglutinable to full titre by a highly potent cholera serum Pasricha and co-authors added, without giving details, that similar changes were brought about by vibriophages grown on three other strains of cholera like vibrios.

As set forth already in the preceding chapter Pasricha and colleagues (1933) claimed that out of 56 strains of "non-agglutinating" vibrios 11 became agglutinable with cholera-immune serum after action of cholera phages while the same result was obtained in 13 instances after the action of vibriophages. Commenting upon these findings Pasricha and his co-workers stated that

> "We have not made a sufficiently extensive study of vibrios and phages in non endemic areas, nor have we made an investigation into the seasonal variations of vibriophages in relation to the epidemic of cholera, to enable us to draw definite conclusions as to the part played by vibriophages in the epidemiology of cholera, but in the laboratory under the influence of a virus disease (choleraphage) the typical cholera vibrio becomes a harmless saprophytic organism which when once again parasitized by another virus disease (vibriophage) assumes the characteristics which render it indistinguishable from the typical virulent type Whether the vibrios regain their virulence or not we have no means of judging. The conclusion is forced upon us that the vibriophages play an important part in the epidemiology of cholera and that they are one of the important factors in bringing about a regeneration of degenerated cholera vibrios."

As reported by Anderson (1940) workers in the King Edward VII Memorial Pasteur Institute in Shillong, bearing in mind that cholera like vibrios found in surface waters might be cholera germs which had lost their specific agglutinability tried to produce a reversion of the organisms to their original state by growing water vibrios in dilutions of antiphage sera. However these attempts gave entirely negative results.

Disquieted by the possibility of diagnostic errors which might be made if haemolytic variants of *V cholerae* were met with in actual practice Bernard and colleagues recommended resorting to serial subculture of possibly modified growths so as to obtain once more colonies of a typical character

Scholtens (1935) inoculated 15 tubes containing 0.5% suspensions of sheep erythrocytes in broth with isolated colonies of a secondary growth of *V cholerae* obtained after phage action Slight but definite haemolysis was noted in those tubes in which undoubtedly as the result of roughening, a flocculent growth took place while haemolysis was almost negligible in the tubes showing a diffuse growth, characteristic of smooth organisms. The variants definitely producing haemolysis were found to be phage resistant but non-lysogenic.

This demonstration of a potentially existing parallelism between S-R transition and aberrant haemolytic reactions given by the vibrios in question is of considerable interest.

While several of the above-quoted observers including Doorenbos, found that under the influence of bacteriophages the specific agglutinability of *V cholerae* might be impaired or even lost, it should now be mentioned that a few workers claimed that strains of this organism, which were no longer amenable to the action of cholera immune sera after they had been exposed to phages, might regain their original agglutinability and that even hitherto "inagglutinable" cholera like vibrios might after phage action show serological reactions identical with those of the true cholera vibrios

As already partly discussed in Chapter 4 claims to this effect have been made by Pasricha, De Monte & Gupta (1931b 1932b 1933)

In their 1931 paper these workers recorded that the lysability of 355 strains of cholera like vibrios of various origin by choleraphages was as follows

Source	*Number of strains tested*	*Type A*	*Type B*	*Type C*	*Type B and C*	*Percentage lysed*
River and tank water	88	—	2	26	18	52.0
Stools of healthy persons	82	—	—	—	13	16.0
Stools of cholera convalescents	126	—	8	12	4	19 0
Totals	296	—	10	38	35	23 0

Note No instance of lysis was observed in the 59 specimens of cholera patients stools, the vibrios isolated from which were frequently found to be contaminated with type A choleraphages.

As noted in the preceding chapter Pasricha and colleagues found that some of the secondary phage resistant colonies which developed after action of choleraphages B C or B and C on these cholera-like strains were agglutinable by a high-titre cholera serum, completely absorbed the agglutinins from this serum, and were capable of producing sera which agglutinated

injections of cholera phage lysate should be either antitoxic or antibacterial or a combination of both. As the bulk of bacterial bodies in a lytic filtrate undergo complete dissolution, one would naturally expect to find in it more of the disintegration products of organisms (the so-called endotoxins of vibrio in this case) than intact bacteria themselves, in addition to bacteriophage protobes which grow at the expense of these organisms Before attributing any property of inducing active antitoxic immunity to such filtrates it must be proved that they contain potent toxins as weak toxins can never act as satisfactory antigens."

In order to test the toxic properties of choleraphage lysates and to explore at the same time the immunogenic value of such preparations, Maitra & Mallick injected three rabbits with 1 to 3-ml doses of a *V cholerae* lysate produced with a potent choleraphage Careful observation of the animals for two weeks showed no impairment of their health. However when the animals were injected intravenously on the 15th day with amounts of killed cholera vibrios equivalent to 1 5 times the calculated minimal lethal dose like the control rabbits they developed diarrhoea, collapsed, and died in 24 hours.

Considering it possible however that addition of choleraphage lysates to standard cholera vaccines might enhance the immunogenic value of the latter six rabbits were given subcutaneously two doses of cholera vaccine of 0.5 ml and 1 ml respectively to which each time 1 ml doses of a cholera phage lysate had been added. Bactericidal tests made two weeks after the second inoculation with the sera of these animals gave results identical with those obtained in the case of a group of control animals, which had been injected only with standard vaccine doses

As far as one is entitled to judge from these limited experiences, addition of phage lysates to standard cholera vaccines does not enhance the bactericidal power of the latter Hence even though it was found that the agglutination titres of the sera of the animals injected with phage lysates as well as with vaccine were on the whole higher than was the case in the control group addition of choleraphage lysates to standard cholera vaccines offers presumably no advantages.

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In a preliminary note published in 1950 Doorenbos & Cossery claimed on the contrary to have transmuted with the aid of an antiphage serum, produced by the repeated intravenous administration of type B cholera phages to rabbits, a rough variant of *V cholerae* Korein into the smooth form.

The two workers stated that they had used for this purpose 1/100 and 1/500 dilutions of the antiphage serum in broth, in which the rough vibrios were cultivated two times in succession for 48 hours respectively Final subcultures were then made and cultures on agar were plated out after an incubation of the broth tubes for 72 hours. After the agar plates had been left at room temperature for 5 days, the vibrios grown repeatedly in the presence of antiphage serum showed—in contrast to those grown under identical conditions in 1/100 dilutions of normal rabbit serum—evidence of a reversion to the S type. Through subcultivation on agar slants growths of a definitely smooth appearance were obtained, which were stable in normal saline and were agglutinated by specific sera at the same titre as the original smooth cultures of *V cholerae* Korein (the causative organism of the 1947 cholera outbreak in Egypt)

While maintaining that the action of the antiphage serum was responsible for the transmutation of rough cholera vibrios into the smooth form, Doorenbos & Cossery admitted the possibility of "a spontaneous R S transformation of the R vibrios when grown on different media" They proposed to confirm the validity of their preliminary findings through further investigations, but—as far as the present writer is aware—no confirmatory publication on this subject has been made Moreover as has been pointed out in the preceding chapter even final proof of such transformations under highly artificial conditions would not constitute proof that analogous reversions take place under natural conditions—a postulation which as has been explained, appears to be altogether unlikely on epidemiological grounds

On the other hand, the evidence adduced above leaves no room for doubt that bacteriophage action is apt to effect a transmutation of typical smooth cholera vibrios into an "inagglutinable" evidently a rough form

Use of choleraphage produced lysates for vaccination

While in order to avoid duplications, the use which has been made of bacteriophages in cholera diagnostic work as well as in the treatment and mass control of the disease will be described in later parts of this book, attention has to be devoted at present to some attempts to utilize cholera phage-produced lysates for vaccination.

As summarized by Maitra & Mallick (1931) it was suggested by d Hérelle, Malone & Lahiri (1930) that a solid and lasting cholera immunity might be produced in man by the administration of single 1 ml doses of choleraphage lysates. Commenting upon this recommendation, Maitra & Mallick stated that it was

"probably based on reported protection against Barbone induced in buffaloes by injections of the phage lysate of *B. bovisepticus* The type of immunity produced by

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Chapter 6

GENERAL PATHOLOGY AND MORBID ANATOMY

PATHOGENICITY FOR MAMMALS

Though, as narrated by Sticker (1912) in his compendium on cholera, it has been repeatedly recorded in the past that large numbers of domestic animals were apt to succumb to cholera when, or immediately before the disease was rampant in man, the validity of such claims has never been substantiated. Emphasizing this lack of trustworthy information, Koch stated in the report he rendered at the 1884 cholera conference in Berlin

"It has been claimed that cholera occurs in cows, dogs, chickens, elephants, cats, and several other animals, but if one scrutinizes these claims, one finds them always utterly unreliable. Up to now we still possess no certain evidence that animals contracted the infection spontaneously at times when cholera was prevalent." [Trans.]

As unanimously held, this dictum of Koch has remained fully valid up to the present, at least as far as the higher animals are concerned.

Koch (1884) further maintained that

"also, all experiments made thus far in animals with cholera substances gave either a frankly negative result or if allegedly positive, were not completely convincing or were not confirmed by other experimentators." [Trans.]

Numerous attempts made by Koch and his co-workers to infect white mice (used in the past with rather questionable success by Thiersch, 1856) by feeding with cholera faeces or with the intestinal contents of cholera victims gave negative results. The same was the case when instead of these animals, other species, particularly monkeys, cats, dogs, or chickens, were tested. Feeding the animals with pure cultures of *V cholerae* was also not successful since the organisms evidently perished in the stomach of the animals instead of reaching the intestines.

To overcome this barrier Koch introduced cholera materials directly into the small intestine of animals after laparotomy and also attempted rectal infection of monkeys, without obtaining success. Administration of

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Yang, Y N. & White, P B. (1934) Rough variation in *V cholerae* and its relation to resistance to choleraphage (Type A). *J Path Bact* 38, 187

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Doyen (1885) found that the resistance of guinea pigs to cholera infection by the oral route could be overcome by previous treatment of the test animals with 40% ethanol at the proportion of 1 7 g per 100 g body weight However Tizzoni & Cattani (1888) who applied both this and Koch s method, were able to produce oral cholera infection in only 4 out of 16 guinea pigs preliminarily given ethanol as against 11 successes in 15 guinea pigs treated according to Koch s method Cultivating the blood of 14 of their successfully infected animals, Tizzoni & Cattani obtained positive results in 7 guinea pigs inoculated according to the latter procedure and only in 2 preliminarily treated with alcohol It also deserves attention that further trials of the alcohol method in rabbits and dogs, to which reference will be made below, did not yield satisfactory results

According to Pfeiffer & Nocht (1889) extensive use was made of Koch s technique in the Berlin Institute of Hygiene for guinea pig experiments with a highly virulent cholera culture which had been isolated in Shanghai When direct use was made of the intestinal contents of animals killed with this culture and the vibrios were passed in this manner through 10 generations of guinea pigs infected orally the virulence of the organisms became so exalted that finally 0 25 ml of the intestinal contents sufficed to produce death in 18-20 hours. Describing the post mortem findings in their test animals, Pfeiffer & Nocht referred in addition to the signs recorded by Koch, to a peculiar pink congestion of the small intestines, a feature also noted by most subsequent observers

As referred to in Chapter 4 Brieger and colleagues (1892) Klemperer (1892) and Pfeiffer & Wassermann (1893) took advantage of Koch s technique to study the problems of cholera immunity in guinea pigs

Sobernheim (1893) also following Koch s technique, found that the introduction of living and heat killed cholera vibrios respectively into the stomach of guinea pigs in equal doses gave identical results He concluded therefore that

"though certainly a multiplication of living vibrios in the intestinal canal takes place, the factor of intoxication plays a considerable role" [Trans.]

The alkali-opium treatment facilitated in his opinion the resorption of the toxins. These postulations of Sobernheim were endorsed by Klemperer (1894), who said that he had already referred to them in his 1892 publication

Sewastianoff (1910) maintained that cholera vibrios, if introduced into the stomach of guinea pigs according to Koch s method in large quantities (5-10 ml of a 24 hours broth culture) entered the blood stream via the intestinal lymph vessels and the mesenterial glands, and thus reached the internal organs He added that in animals which had been infected *per os* i.e. which had been fed with agar-grown cholera vibrios without previous alkalinization of the stomach the organisms could be often demonstrated in the submaxillary lymph nodes, and sometimes also in the blood

laxatives before oral infection likewise proved ineffective Intravenous infection of rabbits with pure cultures of *V cholerae* rendered the animals markedly ill but they recovered. However Koch succeeded in producing fatal infection in white mice through the administration of large doses of cholera cultures by the intraperitoneal route the animals succumbing within 24-48 hours with signs of bacteraemia

As will be described below these rather scanty results obtained by Koch in the course of his work in India have been supplemented by pioneer studies of Nicati & Rietsch (1884a, 1884b) Koch (1885) and Doyen (1885) and subsequently by ample observations of numerous other experimentators The evidence thus made available may be classified as follows.

Infection by the Oral Route

Guinea pigs

Nicati & Rietsch, the first workers definitely able to produce enteric cholera in experimental animals, though mainly relying upon direct intraduodenal infection (see below) found that guinea pigs could be given the disease "even through injection into the stomach with the aid of a tube, if one uses in the latter case a very considerable amount of the poisonous substance"

Likewise using guinea pigs as his test animals, Koch (1885) devised an elaborate method to overcome the barrier created against an entry of *V cholerae* into the intestine by the acid reaction of the stomach juices. He thus described his technique of infection by the oral route

"A preparation of the test animals is necessary for this purpose. They are given first 5 ccm of 5 / sodium carbonate solution and 20 minutes later 10 ccm of broth containing comma bacilli are injected into the stomach Immediately afterwards tincture of opium (1 ccm per 200 g body-weight) is injected intraperitoneally The animals are thus anaesthetized for ½-1 hour but then completely recover" [Trans.]

Koch depicted the results he had obtained with the aid of this procedure in 85 guinea pigs as follows

"On the following day the animals appear to be ill, their fur becomes ruffled, and a marked debility of the hinder extremities and the musculature of the back becomes apparent they die in 1 3 days. At autopsy the small intestines are extended and, like the stomach and caecum, filled with an alkaline, colourless and flocculent fluid, which represents almost a pure culture of comma bacilli." [Trans.]

Though these findings closely resembled those in human victims who had succumbed in the acute stage of cholera, Koch admitted that the rather drastic procedure used by him rendered the guinea pigs also susceptible to infection with the cholera like vibrios of Finkler & Prior (1884) and of Deneke (1885) However the animals were far less amenable to infection with these organisms than to that with *V cholerae* and showed signs different from those produced by the latter

buffer of pH 8, which contained the equivalent of 20 mg dry weight of cholera vibrios.

Subsequently, Freter (1955), working in Burrows' laboratory, established that guinea pigs could be regularly infected with *V. cholerae* by the oral route, provided that (*a*) their normal intestinal flora had been reduced by starvation and inhibited by streptomycin administration and (*b*) a streptomycin-fast strain of *V. cholerae* was used. The detailed procedure adopted by Freter was as follows:

"(1) The animals were starved for 4 days but water was given ad libitum throughout the experiment.

"(2) The second step in the procedure was the administration of 250 mg $CaCO_3$ suspended in 10 ml of distilled water by stomach tube.

"(3) Three hours later the streptomycin-resistant vibrios were given, also by stomach tube, in 15 ml of veal infusion broth (previously diluted with distilled water to half strength) together with 250 mg $NaHCO_3$ and 5 mg streptomycin sulfate.

"(4) One-half hour later 8 mg morphine sulfate were given intraperitoneally to reduce intestinal motility."

Depending upon the dosage used the infected animals showed signs of illness—first listlessness, followed by a gradually developing paralysis of the hind legs, hypothermia, and tremor—after 12-48 hours and death usually occurred within 18-48 hours after infection. At autopsy the caecum, or often the entire bowel was found to be filled with 50-60 ml of fluid containing *V. cholerae* in practically pure culture. On the other hand, cultures from the heart blood and spleen were invariably sterile, thus testifying that the infection was strictly limited to the lumen of the bowel.

A further interesting point was that the lethal doses (LD_{50}) necessary to produce enteric infection in two different races of guinea pigs were markedly at variance—an observation which served in Freter's opinion "as an indication that other mechanisms, beside bacterial antagonism, also play a significant part in determining the resistance of an animal to enteric cholera infection."

In continuation of his studies, Freter (1956a) reported that, by slightly modifying his above-described technique for oral infection of guinea pigs, especially by omitting fasting and morphine administration, he had been able to produce in such animals a long lasting asymptomatic cholera infection, the degree of which could be assessed by performing bacterial counts on the faecal specimens. However rapid disappearance of the cholera vibrios from the stools was found to take place in animals which had been given a streptomycin resistant strain of *E. coli* of human origin together with the *V. cholerae* strain used for their oral infection. Thus, Freter stated, as far as it was possible to draw conclusions from tests made with one strain each of the organisms concerned, his observations showed that bacterial antagonism was a definite factor in the resistance to enteric

in 18-36 hours with lesser doses, survival was prolonged up to 5-12 days at the lower limit of the lethal doses. As far as ascertained, non-fatal infections could be produced with as little as 7 mg (14 000 million vibrios) It was found that during the period of infection

"the animals were listless and obviously ill and became emaciated. There was little or no diarrhoea and the feces continued to be formed emaciation was apparently a result of failure to take food and water At autopsy the capillaries of the small intestine were hyperemic, the tissue somewhat edematous, and in many instances there was shedding of the epithelium. Other than hyperemia of the mesenteric capillaries and a somewhat increased amount of fluid in the peritoneal cavity the remaining gross pathology was essentially that of dehydration and not characteristic. In general then, the fatal disease corresponded closely to that described by Koch (1885)"

Systematic studies in the course of which series of animals were orally infected, sacrificed after various intervals and used for cultivations from the intestines as well as from the peritoneal cavity the liver spleen, kidneys, and adrenals, convinced Burrows and his co-workers that (*a*) cultures from the small intestine were uniformly positive for *V cholerae* while (*b*) cultures from the spleen were positive only in one third to one half of the animals, and (*c*) the other organs were with rare exceptions vibrio-negative. The conclusion reached, therefore was that the infection resulting from oral administration of *V cholerae* to guinea pigs was "confined primarily to the lumen of the bowel with vibrios occasionally reaching the blood stream but taken up in the spleen without further spread" Another important fact established in the course of these investigations through systematic examination of the stools of the infected animals was that

"The infection was shown to be a true infection by demonstration of the multiplication of the vibrios within the animal the total number of vibrios recovered from the feces over the period of infection was as great as 900 % of the inoculum."

A further study by Burrows, Deupree & Moore (1950) on guinea pigs given 100 to 200 r of X radiation and orally infected with *V cholerae* on the following day as well as on a non-infected but irradiated control group showed that in a majority of the infected animals and in about half of the controls a generalized bacterial infection resulted. In the case of the 43 cholera-infected animals examined at autopsy 63 % showed bacteraemia, *V cholerae* being isolated from the heart blood in 48 %, and streptococci or staphylococci in 52 % Commenting upon these findings, compatible with the assumption that a bacteraemia of intestinal origin forms a part of the signs of radiation sickness, Burrows and co-authors pointed out that the cholera vibrio once it had gained an entrance to the tissues, was apt to be pathogenic for the guinea pig

It is noteworthy that Burrows and his co-workers, when making the experiments described above modified their original technique by (*a*) giving to the irradiated animals a short time prior to infection 2 mg of morphine sulfate intraperitoneally instead of opium tincture and (*b*) using as inoculum 2 ml of a suspension of an 18-hour culture in isotonic phosphate

in the same manner 3 other bacterial species (a yeast fungus, a *Sarcina* and an *E. coli* like bacillus) which in Metchnikoff's experience promoted the growth of *V cholerae* on gelatin plates Only half of the animals infected with cholera vibrios alone succumbed to the infection after a considerable delay (6 days or more) whereas out of 22 young rabbits receiving the mixed infection 20 succumbed 36-48 hours afterwards and showed at post mortem signs closely resembling those of human cholera It is noteworthy however that, while cholera vibrios abounded in their intestinal contents, it was impossible to demonstrate the presence of the admixed bacterial species even in animals which had been killed a few hours after infection Metchnikoff's not very satisfactory explanation of this disappearance was that

"one must admit that the role of the infection-promoting microbes is to facilitate [*favoriser*] the first moments of the parasitic existence of the vibrio when the latter has become established, the favouring organisms become useless, so that their disappearance does not prevent the continuation of the choleraic process" [Trans.]

Dealing with the distribution of *V cholerae* in the young rabbits infected orally Metchnikoff stated that

"The cholera vibrio becomes localized principally in the small intestine and penetrates regularly into the large intestine but rarely into the stomach. Outside the intestine, it is most often met with in the gall-bladder In 16 cases examined in this respect, the bile gave vibrio cultures 8 times (50 %) Penetration into the liver is more rare in 24 cases the vibrios have been cultivated 8 times (33 %) Generalization of the cholera vibrios in the blood has been observed in a fourth of the cases studied (12 positive in 48 animals, the heart blood of which was examined)" [Trans.]

Most interestingly Metchnikoff quite often observed instances of cholera in members of his experimental litters which had not been directly infected. He claimed that a contamination of the teats of the mothers was responsible for such contact infections.

Though able to produce a cholera syndrome through oral infection of quite young guinea pigs with the aid of the Massawa vibrio particularly if using this together with the above mentioned infection promoting microbial species, Metchnikoff never succeeded in infecting such animals with his true cholera strain. He stressed in this connexion that the young guinea pigs, because they started to feed on vegetables on the second or even the first day after birth, had a more plentiful flora of intestinal bacteria than young rabbits, which subsisted solely upon the milk of their mothers for several weeks.

Schoffer (1894-95) while fully confirming that quite young rabbits, which fed upon their mothers milk only were highly susceptible to intra-oral cholera infection strongly opposed Metchnikoff's views regarding the role played in this process by other bacterial species.

Schoffer emphasized in this connexion that, in order to cause the disease the vibrios introduced had to possess a high initial virulence instead of

infection. It was possible "that a similar protective mechanism might also be operative in human beings which carry a suitable normal enteric flora "

In a further paper Freter (1956b) adduced evidence that introduction of the above-mentioned streptomycin resistant *E coli* strain also increased the resistance of the guinea pigs to fatal enteric cholera infection. Passive immunization of the animals with OH or O antiserum by the intraperitoneal route as well as oral administration of H antiserum did not protect them *against such infection. However oral immunization with OH or O anti*serum was found to confer protection.

Rabbits

As far as could be established, successful attempts to infect rabbits with *V cholerae* by the oral route were first made in 1894 by Issaeff & Kolle and by Metchnikoff

Issaeff & Kolle established through preliminary experiments on 5 rabbits that it was possible to produce intestinal cholera in these animals, provided that half an hour before administration of the organisms with a stomach tube 10 ml of a 5% solution of sodium bicarbonate were given in the same manner Autopsy findings in the infected animals were similar to those in orally infected guinea pigs with one exception, their blood and internal organs were sterile

Continuing the work with 32 young rabbits, Issaeff & Kolle produced manifest intestinal cholera in 9 while 3 more of the test animals were found to harbour the causative organisms in their intestines The internal organs were invariably sterile Since even the rabbits showing no evidence of successful infection with *V cholerae* had suffered from some diarrhoea and showed at autopsy fatty changes in their liver (as was the case in intravenously infected animals) Issaeff & Kolle assumed that they had recovered from cholera attacks In fact, the serum of one of these rabbits was found to protect guinea pigs against intraperitoneal challenge with *V cholerae*

Prolonged administration of 40% ethanol in doses up to 12 ml did not increase the susceptibility of the rabbits to the above-described method of cholera infection.

Postulating that the inimical action of the intestinal flora in adult animals rather than the acid reaction prevailing in their stomach prevented their infection with *V cholerae* Metchnikoff experimented with 1 to 4-day-old rabbits which received no food besides the milk of their mothers. Part of these animals were intra-orally infected with the aid of a bent glass tube with cholera vibrios only [1] while a second group was in addition given

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[1] It has to be noted that Metchnikoff performed most of the experiments described above with a vibrio from Massawa, the cholera nature of which was rather doubtful. However he obtained identical results with a true strain of *V cholerae* Moreover, the validity of his principal findings was confirmed first by Schofer (1894-95), and then by several other workers including Chookevich (1911; see also Chapter 4, p. 322), who all worked with authentic cholera strains.

6 times in the urinary tract 4 times in the heart blood, 3 times in the "submaxillary glands" and twice in the liver In 3 additional animals killed 6 hours after oral infection cholera vibrios in addition to being found in the intestines were found once in the heart blood the submaxillary glands, and the urine, and twice in the kidneys

Having preliminarily dealt in 1916 with the problem of oral cholera infection of rabbits, Sanarelli published in 1921 an exhaustive study on *this subject*. The salient points of the voluminous conclusions he then reached were as follows

(*a*) The gastric contents of newborn rabbits have a very marked acidity which like that in adult animals, exerts an almost instantaneous bactericidal action upon the cholera vibrios as well as upon other non-spore bearing bacteria In Sanarelli s experience it was impossible to infect quite young rabbits by direct intragastric injection of cholera vibrios after laparotomy

(*b*) The vibrios introduced into the mouth of sucking rabbits do not reach the intestines directly through the stomach but, having been first taken up by the lymphatics, reach the intestinal wall indirectly through the blood circulation

(*c*) The morbid process in young rabbits infected orally involves first the caecum with the appendix and the colon selectively reached by the vibrios. The small intestine is invariably less rich in vibrios, if not entirely sterile, which is the rule for the duodenum. Hence "from the viewpoint of bacteriology as well as from that of morbid anatomy the process in question ought to be considered as a choleraic [*cholériforme*] enterocolitis and not at all as intestinal cholera"

(*d*) The vibrionic enterocolitis may be produced in young rabbits parenterally through subcutaneous intraperitoneal, or intravenous injection of the causative organisms.

In a further publication dealing with the problem of oral cholera infection in adult rabbits, Sanarelli (1923a) held that in these animals also the vibrios deposited on the buccal mucosa could penetrate through the epithelium and could reach the intestines "through the lymph or blood circulation" He admitted however that such invasions took place "with great slowness, some irregularity and in rather feeble proportions" and that reaching the intestines in this manner the organisms produced not an acute, but a chronic process sometimes leading to death from "intestinal cachexia" Old cholera cultures, rich in supposedly more resistant spheroidal forms were most suitable for demonstrating the permeability of the buccal mucosa of adult rabbits by *V cholerae* Sanarelli added that these organisms were also capable of entering the circulation through the mucosa of the nose and the deeper respiratory tract.

becoming virulent afterwards through the action of infection promoting organisms as Metchnikoff postulated Accordingly 7 of Schoffer's young rabbits, *which had remained healthy when infected with an avirulent strain* of *V cholerae* promptly succumbed when afterwards given a highly virulent culture

Schoffer maintained, therefore that one was entitled to assume an infection-promoting role of other bacterial species only in those cases

"where these organisms cause abnormal changes in the intestinal canal, e.g., lesions of the epithelium, which facilitate the invasion of the cholera vibrios, and hence the resorption of their toxin" [Trans.]

He also drew attention to the contention of Issaeff & Kolle (1894) that the frequent occurrence of coccidiosis analogously facilitated the development of cholera in the test rabbits.

Furnishing details regarding 20 of his young rabbits infected intra-orally Schoffer stated that

(*a*) cholera vibrios were invariably present in the intestines, occurring in almost half of the animals in pure culture in the small intestines

(*b*) in the stomach the organisms were demonstrable three times with the aid of peptone water enrichment

(*c*) the heart blood was sterile in 7 instances, and yielded growth of *E. coli* only in two whereas *V cholerae* could be cultivated in 11 instances, but mostly grew scantily occasionally in association with *E. coli*

(*d*) however cholera vibrios could be cultivated invariably from the liver blood, being associated in half of these cases with more or less numerous *E. coli* colonies.

Wiener (1896b) who using Metchnikoff's technique infected several litters of 5-day-old rabbits either with cholera vibrios alone or with both these organisms and *E coli* summarized the findings made at autopsy of these animals thus

"Vibrios were mostly absent from the stomach contents, in which other bacteria were present in varying numbers. The shorter was the interval between infection and death, the more characteristic were the findings in the small and large intestines. In cases running their course within a few hours, the whole intestinal tract was overflooded with vibrios, which were present in dense masses in the whitish flocculi of the caecum, but other bacteria were usually not seen, regardless of whether infection with cholera vibrios only or mixed infection had been used. The more protracted was the course of illness, the more conspicuous were admixtures of other bacteria Invasion of the other organs was not constant. If pure cholera infection was used, the plates inoculated from the liver showed moderate numbers of [*V cholerae*] colonies in two thirds of the cases vibrios were cultivated much more rarely from the kidney but occasionally from the spleen. In the heart blood they could be demonstrated invariably even by smear examination. No other bacteria were found in the internal organs." [Trans.]

Cano (1913) in order to study to what extent oral cholera infection led to a transition of the causative organisms into the internal organs, administered to rabbits less than 20 days old 5 ml of 24-hour broth cultures *per os* In 7 of these animals he demonstrated the presence of *V cholerae*

(usually 7-8 g) and (*b*) as soon as diarrhoea had appeared giving, with the aid of a stomach tube suspensions of 24 hour old agar cultures in 20 ml of 24-hour old broth cultures of *V cholerae*

Positive results were obtained in this manner only with one particularly toxigenic strain and not with another less toxic race That the action of the cholera toxin was of great importance was also shown by the successful use of ether killed growths of the toxigenic strain

If infected with a sufficiently large dose (contents of a whole dish with a diameter of 9 cm) the animals died in 18-48 hours with lesser doses (1/5th 1/10th of such a dish) death took place only after 2-4 days. However rapid results could be obtained when using, instead of culture material small amounts of the intestinal contents of monkeys which had succumbed to the infection

Animals which had been successfully infected soon showed hypothermia and their yellowish diarrhoeic stools were gradually replaced by rice water like stools closely resembling those in human cholera Vibrios abounded in the stool flocculi

At autopsy the usual signs of cholera were found Abundant growths of *V cholerae* could be obtained from the intestinal contents but it is significant that cultures from the heart blood were negative

Sisels

In a report published in 1894 to which reference has already been made in the fourth chapter Zabolotny stated that he had been able to produce an enteric infection in sisels (*Spermophilus guttatus*) by feeding these animals with materials contaminated with cholera cultures or instilling a few drops of a 24-hour-old broth culture of *V cholerae* into their mouths About half of the sisels thus infected succumbed to cholera and still better results were obtained when small amounts of a sodium carbonate solution were added either to the cultures or to the food materials used. Some of the surviving animals, because they had passed through a severe cholera attack, became immune to this infection The fatally infected sisels were found at autopsy to harbour plentiful cholera vibrios in their stomach and intestinal contents. The organisms were also often present in the peritoneal cavity and in the " abdominal organs " (apparently the liver and the spleen) and not rarely also in the blood.

It would be interesting and potentially useful to establish whether this marked susceptibility to oral cholera infection is possessed also by allied species of wild rodents, particularly the ground squirrels abounding in the western part of the USA

Mice

Kamen (1895) stated that he had produced a fatal enteric infection in two commensal (domestic) mice which had been fed with bread contaminated with a cholera suspect vibrio culture The organisms were found to

Assessing the rather unusual postulations made by Sanarelli in regard to the pathogenesis of cholera in rabbits infected orally one must admit that, as will be discussed below parenteral administration of the causative organisms may result in a localization of the infection in the intestines It is further undeniable that direct intra-oral administration of the vibrios may lead to a spread of the infection through the lymph and blood-channels This was definitely proved by Solarino (1939) who was able to produce the disease by depositing material from 24-hour-old cholera cultures on the bucco-pharyngeal mucosa of young rabbits after the oesophagus of the animals had been divided and ligatured at both ends

However irrefutable evidence speaks against the assumption that, as claimed by Sanarelli, an entry of the infection into the intestines *a tergo* and not through the lumen of the gastro-intestinal tract is normally responsible for the causation of enteric cholera. Important as the acid reaction of the stomach is, in experimental animals as well as in man it does not create an impassable barrier against the entry of *V cholerae* On the contrary as has been recorded above several workers have been capable of producing enteric cholera by the introduction of the organisms with the aid of stomach tubes. Further as will be stated below direct intragastric cholera infection after laparotomy has been successfully used. Regardless of whether either of these methods or intra-oral infection of young animals had been resorted to in the experience of most workers a morbid process resembling human cholera resulted, in which involvement of the small intestines was of an obviously initial as well as of a comparatively most serious character A secondary invasion of the blood-stream and subsequently of the internal organs by the causative organs, which were always present and usually abounded in the small intestines, could take place, but occurred in a rather irregular manner and by no means invariably as would have to be a *sine qua non* were Sanarelli s contentions valid. One may claim, therefore that the views of this worker which have been supported by only a few more recent writers, for the last time apparently by Koesoemadilaga (1939) are of historical interest rather than of any actual importance even as far as cholera in experimental animals is concerned

Monkeys

As Mendoza (1913) stated, he had succeeded as early as 1886 in producing cholera in monkeys by infecting them orally after previous alkalinization of their stomach with sodium bicarbonate It could not be ascertained which species of monkeys had been used in these experiments.

Pottevin & Violle (1913) recorded that they had produced cholera both in *Cynomolgus* and in *Rhesus* monkeys by (*a*) administering to these animals sodium sulfate in doses capable of producing copious purging in 3-4 hours

(usually 7 8 g) and (*b*) as soon as diarrhoea had appeared giving, with the aid of a stomach tube suspensions of 24-hour-old agar cultures in 20 ml of 24-hour old broth cultures of *V cholerae*

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be plentiful in the fluid and brownish contents of the enormously dilated small intestines.

Working with some authentic cholera strains, Karliński (1896) was unable to cause infection by feeding in commensal as well as in field and white mice He obtained positive results by feeding commensal mice with Kamen s strain but found that identical tests with field and white mice were unsuccessful. Karliński was rather inclined to assume that Kamen s strain was not of a true nature, but was an aberrant species (*Abart*) of vibrios.

Koesoemadilaga (1939) obtained success when orally infecting white mice with *V cholerae* in 3 different ways, namely (1) feeding the animals with bread which had been moistened with small amounts of vibrio suspensions (2) depositing loopfuls of cholera agar cultures on the tongues of the mice and (3) squirting suspensions of the organisms with the aid of a syringe which had been provided with a blunt needle deep into the mouths of the animals. Since, as has been mentioned before Koesoemadilaga expressed agreement with the views of Sanarelli discussed above it is important to note that only application of the third comparatively drastic, method of oral infection was found to lead to a transition of the cholera vibrios into the blood-stream.

Dogs

Reporting upon experimental cholera infection of dogs, Klemperer (1894) noted that attempts by Nicati & Rietsch (1884c) to produce the disease in this species by the introduction of human cholera dejecta into the alkalinized stomach of the animals had failed. However it deserved attention that Gamaleia (1892) had succeeded in producing in dogs through intravenous injection of cholera vibrios a fatal syndrome characterized by vomiting, diarrhoea with rice water like stools, and collapse Klemperer tried, therefore once more to produce cholera in dogs by administering, after previous alkalinization, with the aid of a stomach tube usually large amounts (50-100 ml) of broth cultures or of suspensions from agar cultures of *V cholerae* and summarized the results of these experiments as follows

Succumbed within about 20 hours (2 after 4½ hours and 2 after 7½ hours respectively)	4
Recovered from a severe cholera attack	1
Had cholera diarrhoea without general signs	7
Negative	13
Total	25

The clinical signs observed in the four fatally infected animals consisted mainly of vomiting, severe diarrhoea (leading in two instances to the voiding of rice-water-like stools), and collapse. At autopsy one could note more or less marked congestion of the mucosa or of all layers of the upper part of the small intestine, and in two cases also congestion of the stomach mucosa. Cholera vibrios were more or less plentiful in the intestinal

contents but were as shown by histological examinations, not present in the walls of the intestines. Desquamation of the epithelium was invariably marked in the upper part of the small intestine

As far as could be gathered from Klemperer s protocols, no generalization of the infection took place He was rather inclined to ascribe the rapid death of the animals referred to above to the action of preformed cholera toxin

Klemperer tried to predispose some of his test animals to cholera infection by preliminary administration of ethanol but reached the conclusion that

"previous treatment with large alcohol doses does not at all reliably create conditions in the intestines under which cholera bacilli cause infection" [Trans.]

Karliński (1896) reported in an article which does not lend itself to exact analysis that by feeding several litters of quite young dogs with cholera contaminated cows milk instead of the milk of their mothers he had succeeded in producing enteric cholera in a minority of these animals. The successfully infected young dogs suffered from diarrhoea and showed at autopsy marked congestion of their small intestines, in which cholera vibrios abounded

Again studying the problem presently under review Sanarelli (1922) admitted that newborn pups, which had not yet partaken of the milk of their mothers were highly susceptible to cholera infection *per os* He found it rather difficult, however to obtain positive results in 24-hour-old dogs and ascribed their resistance to cholera, which became total 36 hours after birth, to the vibriocidal action of the milk of their mothers Sanarelli postulated that in the pups infected orally as well as in young rabbits the cholera vibrios entered the system through the buccal mucous membranes and thus indirectly reached the intestines through the blood-stream. The reasons convincingly speaking against this thesis have been set forth when discussing the production of oral cholera infection in rabbits.

Cats

Success in oral cholera infection of young cats was reported in 1896 by Karliński, whose observations were started several years previously and independently by Wiener (1896a)

The results Karliński obtained when feeding newborn cats with cholera contaminated milk were analogous to those he had recorded in pups infected orally (see above)

Wiener (1896a) used 11 24-hour-old cats belonging to two litters which he orally infected by an unspecified method. All these animals developed severe diarrhoea and voided cholera vibrios in their stools However only one of the 5 animals composing the first litter died 36 hours after infection while the others recovered. The 6 animals of the second litter infected with a different and more virulent strain were all found dead

on the day following infection and half of them showed a most marked congestion of their whole gastro-intestinal tract at autopsy

Cholera vibrios abounded in the intestinal contents of all succumbed animals and were apparently also numerous in their blood, but scanty in the liver and kidneys and absent from the spleen

In view of the postulations of Sanarelli it is important to note that (*a*) the cats infected by Wiener continued to be nourished by their mothers so that at autopsy their stomachs were found to be filled with masses of coagulated milk and (*b*) in one animal at least cholera vibrios could be demonstrated in the stomach contents even though these showed in this as well as in the other kittens a strongly acid reaction.

In confirmation of Karliński s and Wiener s results, viewed with doubt by some writers, Gohar & Makkawi (1948) reported success in the case of two 2-day-old kittens which were infected by soiling the teats of their mother with cholera vibrios and one also by oral instillation of a *V cholerae* suspension. In both these kittens diarrhoea developed 7 days afterwards, and they died 48 hours later showing besides congestion of the small intestines and the abdominal organs, patches of broncho-pneumonia. Cholera vibrios were isolated from the intestines as well as from the stomach and the lungs.

When attempting to infect adult cats as well by the oral route, Gohar & Makkawi were successful in the case of only 2 animals, which had been given large infective doses (500 000 million vibrios in the one case and 25 000 million of *V cholerae* one day after 500 000 million of *E. coli* had been given *per os* in the other) Apparently the infection did not become generalized in these animals.

Direct Intragastric Inoculation

The method of infecting experimental animals by direct injection of *V cholerae* suspensions into the stomach after laparotomy seems to have been used first by Schoebl (1916a) when attempting to produce a carrier state in guinea pigs through the introduction of cholera vibrios by various routes.

Schoebl infected 5 guinea-pigs intragastrically with small doses (" 0.025 of a slant) of a cholera culture possessing presumably a low virulence, giving a suspension of magnesium oxide *per os* before operation. Two of the animals died of pneumonia after 2 and 4 days respectively the others were apparently killed 3-8 days after infection. Positive bacteriological findings were made in four of the guinea-pigs examined 2-8 days after inoculation. Growths of *V cholerae* were obtained twice from the stomach and in all four animals from the gall-bladder as well as from the gastro-intestinal tract.

Experimenting with mostly quite young rabbits, Solarino (1939) was also able to produce cholera infection with the aid of the method presently under review—a result which bears an interesting relationship to the success this worker obtained in young rabbits infected intra-orally after division

and ligature of their oesophagus (see above) For while the latter experiments, made under rather unrealistic conditions showed that *V cholerae* was able to pass into the circulation and to reach the intestines when entering through the buccal mucosa, Solarino s experiences with gastrotomy established the far more important fact that vibrios, if directly introduced into the stomach could pass thence into the intestines in spite of the acidity of the gastric contents. This is all the more significant because it may be claimed that these experimental observations were made under more exacting conditions than those often prevailing in human infection in which for various reasons adduced in Chapter 4 the vibrios may escape the inimical action of the gastric juices

Direct Intestinal Infection

Intraduodenal infection

Nicati & Rietsch (1884b) recorded that

"if after ligature of the common bile duct, one injects into the duodenum of a dog some intestinal contents of a cholera victim or part of a culture of comma bacilli, one finds that the animals die after one or several days and that, as in human sufferers succumbing to cholera after a few hours, the intestinal tract is filled with a pasty milky mass (*Brei*) extremely rich in epithelia. In this, the comma bacilli multiply as markedly as in the stools of human cholera patients after the mass had been exposed to moist air for a certain period, which varies according to the prevailing temperature" [Trans.]

The two workers added that one could obtain identical results in guinea pigs not only without ligature of the bile duct, but even through infection with the aid of a stomach tube They maintained nevertheless that bile even though exerting no untoward influence when added to cholera broth cultures, proved inimical to the vibrios *in vivo* Nicati & Rietsch stated in support of this view that in rapidly succumbing human cholera victims the milky masses which filled the duodenum as well as the ileum and even the opening of the common bile duct, contained not even a trace of bile

Koch reported at the 1885 cholera conference in Berlin that the experiments of Nicati & Rietsch had been successfully repeated by several other workers In his own laboratory positive results had been obtained in 6 out of 10 guinea pigs intraduodenally infected after the ligature of the common bile duct as well as in 13 out of 18 animals in which no such ligature had been made Koch added that, when using this method

the slighter the operation is and the less the intestine is pulled and squeezed when the duodenum is brought in view the fewer positive results are obtained. For this reason a positive result is obtained but occasionally when the abdominal cavity is opened to a slight extent and the injection is made into the first intestinal loop presenting itself instead of into the duodenum. Of the guinea-pigs infected in this manner but one died. Identical tests in 4 rabbits failed to cause death or even illness." [Trans.]

As summarized by Tizzoni & Cattani (1888) some of the early workers successfully using the method of intraduodenal infection had been able to

demonstrate cholera vibrios in the blood of their test animals Finkler & Prior (1885) also claimed to have isolated the organisms from the urinary tract of 4 out of 6 guinea pigs inoculated intraduodenally

Experimenting with rabbits Violle (1914a, 1914b) was unable to obtain positive results by intraduodenal cholera infection even in animals the common bile duct of which had been ligatured. However a young dog, which had remained healthy after imbibing up to one litre of cholera broth could be fatally infected by the intraduodenal injection of but 1 ml of such a culture Further as will be described below, Violle was able to produce cholera in rabbits by introducing the infective material into the small intestine instead of near the pylorus

Arnold & Shapiro (1930) confirmed the negative results obtained by Violle with intraduodenal inoculation of rabbits in so far as they were unable to infect such animals through the injection of saline suspensions of *V cholerae* However the two workers were able to produce a fatal infection, manifested by diarrhoea and the presence of the causative organisms at autopsy in the other internal organs as well as in the intestines, when using, instead of saline a buffered phosphate solution for preparation of the inocula for intraduodenal injection. Arnold & Shapiro (see also Arnold, 1927) maintained in this connexion that in normal animals the contents of the duodenum and the upper half of the small intestine which had a slightly acid reaction were almost free from bacteria, but that abnormal conditions, facilitating bacterial growth, were created there through the production of an alkaline reaction In support of this contention the two workers stated that (*a*) intravenous injection of ¼ MLD of cholera vibrios was innocuous for normal rabbits even if followed by intraduodenal saline injection but that (*b*) a fatal infection could be produced if instead of saline an alkaline buffered phosphate solution was injected intraduodenally even as late as 18 hours after intravenous administration of ¼ MLD of *V cholerae* The animals treated in this manner had cholera vibrios in their diarrhoeic stools and died after 24-48 hours, showing at autopsy findings identical with those observed by Arnold & Shapiro in rabbits infected intraintestinally

Injection into the small intestine

As noted above, Koch (1885) found the method of cholera inoculation into the small intestines only occasionally successful in guinea pigs and not at all effective in the case of some rabbits.

Kolle (1894), referring to 4 guinea pigs, in which intended intraperitoneal infection had accidentally led to an injection of the cholera suspensions into the small intestine stated that only 1 of these animals died spontaneously on the following day of an *E coli* peritonitis, whereas the other 3 remained well until they were sacrificed 24 hours after infection. He

recorded that he had found very large amounts of cholera vibrios in the small intestines of all 4 animals, fewer in the large intestines, only very few in the peritoneal cavity and none in the blood or the internal organs The intestines were in general pale and but moderately filled, thus not showing the characteristic changes met with in guinea pigs which had been orally infected according to Koch's method Hence Kolle maintained, these observations

"proved that the presence of very numerous cholera bacteria is incapable *per se* of producing morbid appearances in the guinea-pig intestine or of producing cholera in these animals. These facts are in accord with the views of R. Pfeiffer that the presence of vibrios in the intestinal lumen is *per se* innocuous as long as the intestinal epithelium remains intact." [Trans.]

However further findings of Issaeff & Kolle (1894) stood in marked contrast to the few results obtained by Kolle under rather unfavourable conditions The two workers injected, after laparotomy suspensions of cholera vibrios into the small intestines of 11 rabbits. Four of these animals, dying at intervals of less than 24 hours to 9 days after infection showed at autopsy usually marked signs of enteric cholera. The causative organisms were plentiful in the intestines of the animals surviving more than a day but were in all four instances absent from the internal organs

A further animal of Issaeff & Kolle's series, which died 15 days after infection had slightly congested intestines, in the contents of which cholera vibrios could be demonstrated with the aid of peptone water enrichment. The other 6 animals showed no or no clear-cut evidence of having become infected.

In contrast to the disappointing experiences he had with intraduodenal infection, Violle (1914a, 1914b) was able to produce enteric cholera in rabbits through injection of small amounts of broth cultures into the small intestine below the entrance of the pancreatic duct, which in this species is situated about 20-30 cm distally from the pylorus provided that either the common bile duct of the animals had been tied off or that the bile secretion had been diminished through intravenous administration of cholera toxin Like Nicati & Rietsch (1884a 1884b) Violle postulated, therefore, that the bile while promoting the growth of *V cholerae in vitro* exerted in the intestine an inimical action on this organism. There was no evidence to show that either a diminution or an increase of the pancreatic secretion exerted an influence on the appearance of enteric cholera in rabbits—a view which had also been held by Nicati & Rietsch

Violle was careful to point out that his experiences with rabbits were not applicable to dogs or monkeys or to man in all of whom the opening of the pancreatic duct was situated in the duodenum near to that of the common bile duct. Experimental cholera infection could be produced in monkeys as well as in dogs through intraduodenal infection following ligature of the common bile duct

Further experiments by Violle & Crendiropoulo (1915) showed that typical lesions could also be produced in rabbits by ligaturing a short loop of the highest part of the small intestine at both ends and injecting into this tied-off portion a suspension of virulent cholera vibrios The two workers found that injection of even enormous inocula into non-ligatured or unilaterally ligatured intestinal loops failed to produce an infection the vibrios disappearing within 24 hours However these negative results stand in contrast to the success obtained by Issaeff & Kolle (1894) with direct injection of *V cholerae* into the small intestines of rabbits. Sanarelli (1921) also stated that he had produced enteric cholera in quite young rabbits with the aid of this method Analogous experiments were made recently by Dutta & Habbu (1955) who were able to produce in 10- to 16-day-old rabbits a disease clinically and anatomically quite similar to human cholera through injection of heavy inocula (100 million of *V cholerae* per 100 g body weight) directly into the small intestine As shown by an examination of 50 young rabbits inoculated in this manner and sacrificed at various intervals, the infection remained almost invariably restricted to the gut, only 3 of the animals showing a vibrionaemia, while a fourth harboured cholera vibrios in the liver The two authors stressed that the "quantitative nature" of the method used by them rendered it suitable for comparative studies on the action of sulfonamides and antibiotics in the treatment of cholera.

In the course of further studies on the pathogenesis of cholera, Sanarelli (1923a) resorted *inter alia* to direct injection of live or killed vibrios into the wall of various parts of the intestine of rabbits or into the plaques of Peyer and other lymphatic tissues of the intestines. The conclusions to which these experiments led were as follows

(*a*) "Direct injection of live or killed [cholera] vibrios into the intestinal walls of rabbits leads only to tardy death from marasmus or intestinal cachexia — conditions associated frequently with secondary infections with pyogenic bacteria or *B coli*

(*b*) "More serious on the contrary is the direct injection into the lymphoid tissues of Peyer's plaques, of the appendix and the *sacculus rotundus* In such cases death may occur a short time (12 18 hours) after infection or several days later One finds at autopsy an enteric process involving the whole intestine, which may be very acute, subacute, or chronic. Often there are also typical renal lesions with albuminuria

(*c*) "The vibrios injected in suitable doses into the major lymphatic organs of the rabbit intestine spread immediately in large quantities along the walls of the digestive tube and into the blood, reaching the buccal cavity and the gall-bladder and causing a most severe and fatal enteritis with diffuse epithelial desquamation. In this case one finds the vibrios in the contents of the whole digestive tube as well as in the stomach walls If death takes place late one finds at autopsy the usual appearances of intestinal cachexia, which sooner or later favours the development of one of the usual secondary infections. [Trans.]

As recorded in the 1946 report of the Indian Research Fund Association the method of injecting *V cholerae* suspensions after laparatomy into the

small intestine had been resorted to in the case of monkeys in the King Institute, Guindy Madras. To lower the resistance of the animals to cholera, either 20 ml of hot distilled water (56°C) were injected into the gut, or deep X ray irradiation was used one week before infection In 5 out of 6 monkeys which had survived irradiation diarrhoea became manifest 48 hours after infection but apparently cholera vibrios could be found in the stools for a few days in two instances only and no spontaneous death from cholera took place. In the animals which had been sacrificed, a slight congestion of the omentum and some histologically manifest desquamation of the mucosa of the small intestine could be noted

A technique analogous to that of Violle & Crendiropoulo (1915) was recently used by De & Chatterjee (1953) to study the action of *V cholerae* on the intestinal mucous membranes. Both adult rabbits and rats were used for this purpose, but only the former were found satisfactory because in rats the contents of the isolated loops of the small intestine were almost invariably free from vibrios 24 hours after 1 ml doses of freshly made peptone water suspensions of young cholera cultures had been injected. However interesting results were obtained in a series of 10 analogously treated rabbits.

While in control animals, which had been injected with sterile peptone water the isolated intestinal segments were collapsed and empty in the infected rabbits they were distended with fluid and swollen to the diameter of a thumb. The vessels of their walls were congested. The fluid contained in the isolated segments was usually rice-watery with a pinkish hue and contained cholera vibrios, whereas these were absent elsewhere in the small intestine. Microscopically shreds of mucus with numerous epithelial cells were seen in the fluid, but pus cells were rare and macrophages altogether absent. The albumin content of the fluid was invariably high, ranging from 1 / to 3 8 /.

The most prominent changes found upon histological examination in the isolated intestinal segments of the cholera-infected rabbits were marked oedema and widening of the submucosa of the wall. The lymphatic channels appeared to be enlarged, the larger blood vessels were engorged. The summits of the villi were often necrotic, but there was nowhere any evidence of a cellular infiltration.

Considering the findings described above and also noting that Evans blue solution injected intravenously leaked into the contents of the ligatured intestinal segments, De & Chatterjee concluded that *V cholerae* altered the permeability of the intestinal capillaries to proteins.

Gupta and co-workers (1956) obtained results closely resembling those just described when injecting cholera cultures into isolated loops of the rabbit intestine. It is of great interest that they also observed similar manifestations when instead of *V cholerae* growths some of the strains of cholera like vibrios isolated during a mild gastro-enteritis outbreak at Allahabad in 1954 (see Yajnik & Prasad, 1954) were used for such tests Commenting upon these findings the authors suggested

" that many so called inagglutinable strains are capable of producing clinical cholera. But the antigenic structure and choleragenicity of vibrios are independent attributes and

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Direct Gall-bladder Infection

Violle (1912) apparently the first worker to use the method of injecting after laparotomy cholera vibrios directly into the gall bladder of experimental animals, did so not with the aim of producing infection but in order to study whether this method of administering *V cholerae* was apt to immunize rabbits. He avoided, therefore, the use of massive inocula which were apt to kill these animals

Violle found that only slight general and local reactions resulted when non lethal doses of cholera vibrios were injected into the gall bladder of rabbits after the bile had been removed and the bladder had been washed with alkaline water but without preliminary ligature of the common bile duct. The vibrios were apt to survive in the gall bladder of these animals for 10-15 days, but no generalization of the infection took place and no state of immunity was engendered.

If cholera vibrios were injected into the gall bladder of rabbits prepared in an identical manner, after the common bile duct had been tied the organisms first multiplied and a marked local reaction was produced, characterized by congestion of the gall-bladder mucosa and epithelial desquamation. Leucocytes soon began to accumulate and, as assumed by Violle, were responsible for the destruction of the vibrios which soon took place. As proved by serological tests, a marked formation of antibodies resulted

Schoebl (1916a) to whose investigations reference has been made already earlier in this chapter used direct inoculation into the gall-bladder of guinea-pigs (without preliminary ligature of the common bile duct) as one of several methods to produce a cholera-carrier state in his test animals. Comparing the results he obtained in this manner with those of infection by other routes he found that

"Direct inoculation into the gall bladder stomach and small intestine and inoculation by feeding proved successful inasmuch as a certain percentage of the inoculated animals were found to harbor cholera vibrios in the alimentary canal. This was ascertained by bacteriological examination of various parts of the digestive system, made in the great majority of cases immediately after death.

"The intravesicular inoculation (i.e. gall bladder infection) proved to be far superior to other methods. Practically every one of the animals inoculated in this way harboured cholera vibrios."

In the course of a further study on the production of a carrier state in experimental animals, Schoebl (1916b) used a cholera strain the virulence of which had been exalted through gall bladder passage repeated several times. Most of the guinea pigs "intravesically" infected with these virulent organisms were decidedly ill after inoculation and some died 3-4 days after such injections had been made

The animals which had died spontaneously showed at autopsy a more or less extensive swelling of the abdomen, due to a haemorrhagic oedema

may be dissociated. Hence to define true cholera vibrio a search for the mechanism and the test for choleragenicity alone would provide the decisive answer"

The present writer for one finds it impossible to agree with this sweeping statement. The fact that, as apparently confirmed by Gupta and colleagues, cholera like vibrios may occasionally prove pathogenic does not entitle one to consider such strains *choleragenic* On the contrary all that can legitimately be said of them is that they appear to be capable of causing *choleraic* syndromes. To make the latter faculty instead of the result of serological tests the decisive factor in classification of the vibrios would be quite unjustified. In fact, were one willing to follow the lead of Gupta and his co-workers one would be unable to draw a sharp line of distinction between the vibrio group and other bacterial genera, particularly the salmonellae some of which are occasionally capable of producing syndromes clinically quite indistinguishable from cholera gravis

Intrarectal infection

As far as could be established, the method of producing cholera in experimental animals through the introduction of the causative organisms into the rectum has been utilized by only a few workers, of whom the first, according to Sewastianoff (1910), was Nasaroff (1907) whose publication was not accessible to the present writer

Sewastianoff (1910) obtained positive results with the method presently under review in guinea pigs and in rabbits. He stated that the intrarectal introduction of large doses of cholera vibrios led in the case of the former animals to the appearance of these organisms together with *E coli* in the internal organs. Analogously he maintained that

"If one introduces large quantities of cholera vibrios through the anus and subsequently closes this with collodion according to the method of Nasaroff and Jurgelunas, one can produce in rabbits a fatal mixed infection, in which the cholera vibrios as well as the other intestinal bacteria penetrate into all internal organs and the animals die in 1 2 hours. It is not possible to produce the infection without occlusion of the anus with collodion." [Trans.]

In agreement with these findings, Crendiropoulo (1921) noted that the intrarectal administration to rabbits of the 10th or even the 25th part of a cholera slant led in 15 minutes to an appearance of the organisms in the intestine as well as in the blood, bile stomach, and urine the vibrios persisting in the body of the animals for 24-36 hours.

Sanarelli (1921) found it impossible to produce a fatal infection in quite young rabbits by intrarectal introduction of cholera vibrios. However examining animals which had been sacrificed a few hours after such inoculations, he noted that the organisms had spread in the intestinal tract as far as the jejunum.

were obtained from the bone marrow as well as from the site of the infection However, in 27 tests in which *V cholerae* was applied to or rubbed into, the intact (unshaved) skin the presence of the organisms could never be demonstrated in the heart blood or the organs and 4 times only at the site of the infection

As far as one is entitled to judge from these findings which do not seem to have been supported by further observations *V cholerae* appears to be incapable of penetrating through the intact skin

Intranasal and intratracheal infection

Besides resorting to intravenous infection Diatroptoff (1894) inoculated *V cholerae* into the nostrils or, after tracheotomy into the trachea of rabbits.

All 12 animals, into each nostril of which 1 2 loops of a cholera culture had been placed, succumbed within 5-20 days after having suffered from diarrhoea and emaciation In the 8 instances in which death had taken place not later than on the 9th day cholera vibrios could be isolated in nearly pure culture from the fluid intestinal contents, but not from the blood or the internal organs.

The rabbits infected intratracheally with 2 3 loops of a *V cholerae* culture usually succumbed after 3-4 days, yielding positive cultures from their blood and organs as well as from the intestinal contents. In the animals which survived somewhat longer, cholera vibrios were found to be present only in the intestinal contents and even these gave negative results if death had taken place 15-20 days after infection.

Subcutaneous infection

The method of subcutaneously inoculating experimental animals with *V cholerae* appears to have been used by Nicati & Rietsch who as stated by Pfeiffer (1892) without further details, " had succeeded in poisoning guinea pigs by the administration of live cholera vibrios into the subcutaneous tissue " The French publication (1884c) in which Nicati & Rietsch presumably recorded this result was not available to the present writer

Koch (1885) found that guinea pigs which had been inoculated subcutaneously or intraperitoneally with specially toxigenic cholera strains usually succumbed within a few hours, after having shown signs of collapse similar to those which developed more slowly in animals infected orally He ascribed therefore, the death of the guinea pigs infected parenterally solely to an action of the cholera toxin On the other hand, some other early observers, enumerated by Tizzoni & Cattani (1888) finding that their subcutaneously inoculated test animals, which died after only 12 24 hours, harboured *V cholerae* in the peritoneal cavity as well as in the internal organs, assumed the presence of a generalized cholera infection.

A fibrinous exudate was present in the peritoneal cavity The internal organs were congested the spleen also enlarged and soft. Cholera vibrios could be recovered by culture from the subcutaneous oedematous fluid, the peritoneum, the arterial blood, and the internal organs.

In the far more numerous animals which did not succumb to the infection but were sacrificed at different intervals, various degrees of inflammation were found in the gall bladder Pathological changes were also found to a varying degree in the liver but seemed in part at least of a chronic character instead of being the result of the infection. Except in the animals examined later than 14 days after inoculation, cholera vibrios were invariably found in the intestinal contents as well as in the gall bladder However the organisms were absent from the peritoneum, the blood, the spleen and the lung, and were but occasionally found in the liver

Commenting upon the results of identical tests made in rabbits, Schoebl stated

"With regard to the intravesicular inoculation the rabbits behaviour differed from that of the guinea-pig inasmuch as the inflammatory process which followed the injection of cholera vibrios brought about the occlusion of the gall-bladder so that the cholera vibrios were no longer to be found in the intestine at a time when they were still present in the contents of the gall-bladder

"It is evidently a benign process which shows marked tendency to healing i.e. the cholera vibrios disappear from the animal s body and the animal survives, altho a large percentage of the animals showed signs of chronic intoxication."

In contrast to these experiences of Schoebl Calvano (1933) found the method of administering small doses of cholera vibrios directly into the gall-bladder of rabbits most suitable to produce in these animals the features of human cholera. Injection of double doses into the small intestine of control animals as well as intraperitoneal administration of whole agar slants of *V cholerae* proved in Calvano s experience unsuccessful.

Parenteral Infection

Percutaneous infection

Following up preliminary observations by a few Japanese observers, Matsumoto Ando & Shiraiwa (1927) experimenting with guinea pigs, systematically studied the problem of the permeability of the skin by *V cholerae* They applied for this purpose suspensions of the organisms in peptone water or tap-water to the shaved or the intact skin of their test animals and killed them 15-27 hours later Cultivations were then made immediately from the site of the infection as well as from the blood and the internal organs. In 4 out of 10 guinea pigs to the shaved skin of which suspensions of cholera vibrios had been applied, it was possible to cultivate the organisms, not only from the site of the infection, from the heart blood, and from various internal organs, but also three times from the bone marrow and twice from the intestines In a fifth animal positive results

In view of this postulation it is not surprising to find that Issaeff (1894) described the strain Pfeiffer had given him for immunological studies as possessing

"all properties of a typical cholera microbe, being pathogenic for guinea-pigs and rabbits if administered intraperitoneally but not upon subcutaneous inoculation and being not pathogenic for pigeons in doses of one loop" [Trans.]

Similarly Pfeiffer & Issaeff (1894) characterizing the strain they used for further immunological work, stated that this was lethal for guinea pigs infected intraperitoneally in doses of 1/10th-1/12th of a loop with a capacity of 2 mg, but produced if given subcutaneously in quantities of one loop only passing fever and a local reaction terminating in skin necrosis.

Kolle (1894) in a study on experimental cholera in the guinea pig, maintained that the method of subcutaneous infection required "very large inocula to kill the animals and was comparatively unreliable" He further stated that after subcutaneous (or intraperitoneal) injection

"one finds the cholera vibrios in the blood of guinea-pigs only under quite peculiar experimental conditions, but never if autopsy is made soon after death, in such amounts that one could speak of a fatal septicaemia The conditions under which a transition of the vibrios into the blood via the lymph channels takes place are, firstly doses which, in relation to the body weight of the animals, are multiples of the absolutely lethal doses Secondly the vibrios are sometimes demonstrable in the blood, when the agonal period was prolonged. In this case they are found particularly often in the blood of the right ventricle, while they are absent from the blood of the left ventricle and the organs. " [Trans.]

The findings Kolle made regarding the transition of the cholera vibrios into the intestine will be considered together with his observations on intraperitoneal infection.

In a supplementary study devoted to investigations on experimental cholera in rabbits, Issaeff & Kolle (1894) stated that subcutaneous inoculation of such animals with small doses led neither to local reactions nor to general and intestinal manifestations after 24 hours the organisms were no longer demonstrable at the site of the infection If large doses were used, there resulted merely a local reaction consisting of the formation of an abscess, in the pus of which the vibrios were demonstrable Such animals were apt to succumb but even then the organisms could not be found in the blood or in the organs

In contrast to the observations recorded above Hahn (1905) found that 2 out of 4 Russian cholera strains which had been fairly recently isolated produced a rapidly fatal septicaemia in rabbits if administered in ¼-loop doses either by the subcutaneous or the intraperitoneal route In the case of the other 2 strains somewhat higher doses were necessary to obtain positive results by subcutaneous inoculation. As asserted by Hahn there was no reason whatsoever to doubt the authenticity of these strains.

Of particular interest is the fact that Babes (1885), one of the workers quoted by Tizzoni & Cattani found that 1 out of 2 white mice, which rapidly succumbed after injection of cholera vibrios into the root of their tails, showed moderate numbers of the organisms in the fluid whitish contents of its intestine as well as in the blood and the spleen. Analogously Buchner (1885) recorded that subcutaneous injection of a guinea-pig with an atypical vibrio strain, which had been isolated during a cholera outbreak at Naples, led to a congestion of the middle portion of the small intestine and to the appearance of numerous "comma bacilli" there and more still in the lower portion of the small intestine Observations identical with that of Buchner were recorded by Cunningham (1887) who no doubt worked with typical cholera strains

While unable to produce a fatal cholera infection in normal guinea pigs through subcutaneous inoculation Tizzoni & Cattani (1888) obtained positive results when using this method in combination with either intra peritoneal injection of opium tincture or administration of ethanol with the aid of a stomach tube A large majority of the animals thus infected harboured *V cholerae* in their blood, while cultivations from the peritoneal cavity made in three instances only also yielded positive results.

Further advantage of the method of subcutaneously inoculating guinea pigs was taken by Pfeiffer (1889) for a study of a cholera like vibrio (*V metchnikovi* Gamaleia, 1888) He found that this organism, if administered subcutaneously in adequately large doses, produced a generalized infection resulting in death after 18-24 hours However while the organisms abounded at the site of the infection as well as in the blood and the internal organs, they were scanty in the contents of the intestines, which showed no gross changes.

Vincenzi (1892) found that subcutaneous administration of one drop of a cholera broth culture sufficed to produce in guinea pigs an abundant oedema at the site of inoculation and to kill the animals in 20-30 hours. Since, however he worked with cultures from Massawa, the true nature of which is dubious, these findings have to be considered with scepticism. Be this as it may Pfeiffer (1894) working with authentic fresh cholera strains, established that most of these cultures,

"regardless of whether they had been derived from the most severe, rapidly fatal cholera cases or from slight infectious diarrhoeas, showed a remarkably uniform behaviour The *dosis lethalis minima* for intraperitoneal infection was invariably not more than a fraction of a loop—usually $\frac{1}{6}$–$\frac{1}{8}$ of a loop sufficed to kill the guinea-pigs. On the other hand, the subcutaneously inoculated guinea-pigs reacted merely with fever whereas pigeons survived the infection." [Trans.]

An exception was formed by 3 strains only which in the doses mentioned above killed all guinea pigs infected subcutaneously and some of the pigeons. On account of this unusual behaviour Pfeiffer was inclined to doubt the authentic nature of the 3 strains.

vibrios were injected To judge by two protocols given by this worker, the animals in question one of which died 12 hours after subcutaneous inoculation, while the other was sacrificed after about the same interval showed vibrios only at the site of the infection and in the intestines but not in the blood, bile, or peritoneal cavity

Suzuki (1926) who infected guinea pigs by various routes with an El Tor strain *(V kadiköj)* found that subcutaneous administration of these organisms led to a generalized infection with the appearance of the organisms in the blood, peritoneum, liver spleen, kidneys, and bone marrow but not in the stomach small intestine and urine

A valuable study on the subject presently under review was made by Ray (1927 see also the preliminary communication by Hahn, 1926) who for this purpose infected guinea pigs subcutaneously intraperitoneally or intracardially either with old stock cultures of *V cholerae* or with freshly isolated strains. It was found that, regardless of which mode of infection was used, inoculation with the stock strains led but exceptionally to an appearance of the organisms in the blood the intestines, or the internal organs. On the other hand, positive bacteriological findings were obtained from these substrates with great regularity when freshly isolated cholera strains were used for parenteral infection

Ray refuted the idea that an invasion of the various organs by *V cholerae* took place merely during the agonal period, because he was able to obtain positive blood cultures not only in the case of animals succumbing spontaneously but also in those which had been sacrificed and had been examined immediately afterwards. He admitted however that the infective doses used by him (even in the case of the freshly isolated strains, usually $\frac{1}{6}$ of a slant) were very large " and thus not in accord with natural conditions "

Reporting on a few additional observations in guinea pigs infected subcutaneously or intraperitoneally with *V metchnikovi* Ray stated that these organisms could be found in the blood and the intestines if similarly large inocula ($\frac{1}{6}$ of a slant) were used. He concluded from these and the findings recorded above that " there were no principal differences in the septicaemic properties shown respectively by *V metchnikovi* and *V cholerae* "

A further most interesting observation made by Ray was that the majority of guinea pigs which had been subcutaneously infected after their common bile duct had been ligatured did not show cholera vibrios in their intestine At first glance these findings indicated that *V cholerae* introduced parenterally entered the intestines with the bile However after 5 ml of ox bile had been given *per os* the organisms could be demonstrated in the intestines of the animals infected after ligature of the bile duct. It appeared, therefore, that the vibrios reached the intestines through the blood-vessels and not through the bile Ray added that

Results analogous to those of Hahn were obtained by Kabeshima (1918a) through subcutaneous infection of white mice with 1 El Tor and 2 classical *V cholerae* strains respectively all of which proved lethal to the animals if administered in doses of $\frac{1}{50}$ $\frac{1}{150}$ of a loop However while emphasizing that these uniform findings spoke in favour of the cholera nature of *V El Tor* Kabeshima pointed out that his cholera strains, which produced " haemotoxin ", probably possessed an exalted virulence.

Further studies on subcutaneous inoculation of experimental animals with *V cholerae* were undertaken almost exclusively with the aim of ascertaining to what extent this as well as other modes of infection led to its generalization and its localization in the intestines—problems which will receive further consideration later in this chapter Record has to be made at the present juncture of the following observations

Crendiropoulo (1921) found that subcutaneous administration of *V cholerae* usually did not lead to a dissemination of the infection Even if lethal doses were given only 1 out of 12 rabbits showed a presence of the organisms in the intestinal tract (appendix) The test animals appeared to succumb to toxaemia rather than to septicaemia.

On the other hand Sanarelli (1921) subcutaneously infecting quite young rabbits, found ample evidence for a generalization of the infection and its localization in the intestines.

Sanarelli noted in particular that subcutaneous inoculation of 4-day-old rabbits led to (*a*) an inflammatory process at the site of infection, where vibrios abounded (*b*) appearance of the causative organisms in limited numbers in the blood, liver spleen, and duodenum (*c*) presence of most numerous vibrios accompanied by that of desquamated epithelia in the diarrhoeic contents of the markedly congested small intestines and also in the large intestine

In somewhat older e g., 6-day-old, rabbits the morbid appearances resembled more closely those considered typical for enteric cholera by Sanarelli Vibrios appeared to be rather rare in the blood as well as in the duodenum and the upper part of the small intestine, but were most plentiful in the caecum and the colon

To judge from a short remark in Sanarelli s paper he found that subcutaneous inoculation of *guinea-pigs* led after a more prolonged course of illness to marked signs of cholera in the intestinal tract.

Using guinea pigs, Masaki (1922) found that

" the virus injected subcutaneously remains at first during 2 3 hours in the cellular tissue. The leucocytes assemble at the site of inoculation and form an abscess. A part of the vibrios leaves the subcutaneous tissue and progresses towards the intestinal mucosa six hours after inoculation one already finds the vibrios in the intestine." [Trans.]

Masaki noted that abscess formation at the site of inoculation took place with particular regularity when massive doses of little virulent cholera

The validity of this contention was vigorously denied by Vincenzi (1887) who stated that he had obtained entirely negative results in 17 guinea pigs infected intraperitoneally with the aid of glass capillaries cautiously introduced after laparotomy However this worker found that if the intestine was mechanically irritated before infection

"regardless of whether they had been introduced into the peritoneal cavity or into the lung or subcutaneously or directly into the blood vessels, the comma bacilli enter by means of the capillary haemorrhages, invariably forming at the site of the irritation into the intestinal lumen, find there favourable conditions for development and cause the death of the animals." [Trans.]

Vincenzi added that presumably identical results might be obtained through chemical irritation of the intestines.

In marked contrast to these statements Vincenzi (1892) maintained that the blood as well as the intestines of intraperitoneally or intrapleurally infected guinea pigs invariably contained viable cholera vibrios It is noteworthy however that—as noted above (page 422)—he used Massawa strains of doubtful authenticity for his second series of investigations

Be this as it may Pfeiffer (1892) in his initial study on the toxin of *V cholerae* emphasized that he had never been able to find the organisms in the intestinal contents of intraperitoneally infected guinea pigs He added that

"the peritoneal contents and the blood were found sterile several times even if autopsy was made immediately after death In the majority of the cases, scanty *V cholerae* colonies could be cultivated from the peritoneal exudate, but even then the blood was sterile. In 2 3 cases the peritoneum contained numerous viable cholera vibrios and then scanty vibrios could be demonstrated in the heart blood." [Trans.]

Pfeiffer concluded from these observations that, since as a rule the intraperitoneally injected cholera vibrios were rapidly killed, one had to ascribe the death of the test animals not to an infection, but to an intoxication He admitted, however that, when small inocula were used, the organisms rapidly multiplied at first, until the amount of transudated serum became sufficient first to impede and then to prevent their further development. In support of his contentions he adduced ample evidence to show that intraperitoneal administration of killed cholera vibrios in appropriately increased doses was as lethal for guinea pigs as injection of live organisms

In strict contrast to Pfeiffer s findings, Gruber & Wiener (1892) insisted that intraperitoneal injection of killed cholera cultures was almost or even totally ineffective even in amounts 20-30 times larger than the lethal doses of live vibrios Since, on the other hand in the experience of these two workers, intraperitoneal injection of young virulent cholera cultures led to a most marked multiplication of the vibrios not only in the peritoneal exudate but even in the pleural cavity Gruber & Wiener postulated that

it was impossible to decide whether (*a*) the bile successfully used for bacterial enrichment *in vitro* exerted an analogously favourable action in the intestines, or (*b*) as was the case in oral vaccination, the bile facilitated the passage of the organisms through the intestinal wall or (*c*) unknown factors were at work. The present writer believes that an intestinal irritation produced through bile administration might be of importance in this respect. As stated in Chapter 4 bile administration for the purposes of oral cholera vaccination has been found capable of exerting such an irritating effect.

Reference has to be made finally to the observations of Panja & Paul (1943) who made a comparative study of the invasiveness of (*a*) true cholera vibrios (*b*) "paracholera" vibrios i.e., cholera like organisms isolated from patients with clinical features of cholera and (*c*) water vibrios isolated from the Hooghly river at Calcutta. When examining the blood of their test animals, obtained from the skin of their ears 2, 18 and 36 hours after subcutaneous inoculation, Panja & Paul found that an early invasion of the blood stream was effected only by the true cholera vibrios and by one of the two "paracholera" strains used, but not by any of the water vibrios, which could be demonstrated in the blood but occasionally even 36 hours after infection. Nevertheless, 75% of the test animals infected with the water vibrios succumbed, as against a mortality of 50% caused by the cholera vibrios and a death rate of 62% in the case of "paracholera" infection. At autopsy the animals invariably showed congestion of the small intestines which were abundantly filled with a whitish glairy fluid. The causative organisms could be isolated from there as well as from the blood the peritoneal cavity and the gall bladder

Panja & Paul found that the 3 kinds of vibrios used by them showed no marked differences in pathogenicity when administered orally to guinea pigs or intravenously to rabbits

Intraperitoneal infection

As summarized by Baumgarten (1921) the method of intraperitoneally infecting white mice with *V cholerae* originally used by Koch (1884—see above page 398) also proved successful in the hands of a few other early observers. Positive results with this mode of infection in guinea-pigs seem to have been obtained first by Hueppe (1887) who found that intraperitoneal administration of 1 ml or even of 1 drop of cholera broth cultures usually killed these animals within 24 hours, sometimes at longer intervals up to 5 days. He noted that their small intestines showed a congestion identical to that met with in intraduodenally infected guinea pigs and that their fluid intestinal contents abounded in cholera vibrios In Hueppe's opinion the presence of the organisms there was the result of their direct entry into the intestine through "preformed stomata of the serosa"

Stage IV the peritoneal exudate was almost clear and contained besides most numerous cholera vibrios only few leucocytes and small numbers of erythrocytes.

Observations on 69 intraperitoneally inoculated guinea pigs and groups of such animals infected with *V cholerae* with the aid of other methods led Kolle (1894) to the following main conclusions

(*a*) Cholera vibrios appeared in the intestines of intraperitoneally injected guinea pigs in large numbers only when the intestines had been accidentally punctured.

(*b*) If, on the contrary, inoculation had been made properly, the organ isms were absent from the intestines in 80% of the animals, while they were so scanty in the remainder of the guinea pigs as not to be demonstrable in smears.

(*c*) Results analogous to those just recorded were obtained with the aid of methods precluding an accidental lesion of the intestines, such as subcutaneous or intrapleural injection direct introduction of the organ isms into the circulation or after laparotomy into the peritoneal cavity

(*d*) While there was no doubt that the vibrios reached the intestines with the blood stream, it could not be decided with certainty whether they actually entered the intestinal lumen or were present merely in the capillaries of the mucosa

(*e*) It was possible to produce through intraperitoneal administration of suitable doses a fatal disease in guinea pigs, in which the cholera vibrios remained absent from the blood, the intestines, and the internal organs Even the peritoneal cavity remained sterile if minimal lethal doses had been given The process of intoxication thus induced in the animals was the analogon of the algid stage in human cholera.

Results closely resembling those of Kolle were recorded by Klemperer (1894) who postulated that the process induced through intraperitoneal cholera inoculation in guinea pigs was essentially an intoxication because (1) the appearance of signs and the occurrence of death were extremely rapid (2) killed vibrios produced an identical syndrome and (3) living vibrios were apt to cause a fatal disease without entering the circulation. Klemperer stated in the latter respect that 16 attempts to cultivate *V cholerae* from the heart blood of his test animals 2 attempts to obtain positive cultures from the spleen as well as 5 attempts to demonstrate the presence of the organisms in the intestine of sacrificed animals gave entirely negative results

Issaeff & Kolle (1894) obtained results similar to those recorded by Kolle in guinea pigs through the intraperitoneal inoculation of young rabbits in the animals injected with large doses, the vibrios abounded in the peritoneal cavity were scantily present in the blood, and the internal organs, and sometimes occurred in quite small numbers in the intestines

this mode of inoculation produced a specific infection and not an intoxication of the test animals

The conclusions reached by Sobernheim (1893) through an examination of 24 intraperitoneally infected guinea pigs were that (*a*) cholera vibrios were invariably present in the peritoneal exudate and usually abounded there (*b*) in about two-thirds of the animals the vibrios were scanty in the blood or even altogether absent, whereas they were plentiful in 7 instances (*c*) in 20 of the animals, the intestines contained most numerous cholera vibrios often in practically pure culture

Sobernheim found, however, no evidence to show that the organisms penetrated directly from the peritoneal cavity into the intestines. Refuting Hueppe's postulations he maintained, therefore, with great reason that the cholera vibrios, initially "resorbed" by the lymph vessels, were further transported by the blood stream In his opinion, however in experimental cholera the blood served merely as a transport vehicle for the causative organisms and not as a substrate for their multiplication, as was the case in septicaemic processes caused by other bacteria.

Confirming Pfeiffer's observation that

"it was possible to kill the animals with adequately increased amounts of killed cultures with appearances identical with those obtained through injection of living cultures" [Trans.]

Sobernheim maintained that the process induced through intraperitoneal administration of the latter was not of a purely infectious nature, but that "the toxic moment also played a considerable role"

Further investigating the problem presently under review, Pfeiffer & Wassermann (1893) drew a distinction between four stages or one should rather say degrees of the process induced in guinea pigs through intraperitoneal cholera inoculation

(1) Minimal amounts of cholera vibrios produced merely passing fever (Stage I)

(2) Somewhat higher doses led after a short febrile period to a marked lowering of the body temperature and other signs of collapse, from which, however the animals recovered within 24 hours (Stage II)

(3) Administration of carefully increased doses so as just to reach the minimal lethal dose led to a fatal cholera intoxication but if autopsies were made immediately after death, the peritoneal cavity was sterile or contained only solitary cholera vibrios, which were usually enclosed in leucocytes (Stage III).

(4) If finally massive doses corresponding to those used by Gruber & Wiener were given, the peritoneal cavity contained most numerous vibrios (Stage IV).

Pfeiffer & Wassermann added that, whereas the animals in Stage III showed at autopsy a fibrino-purulent exudate in their peritoneal cavity in

Stage IV the peritoneal exudate was almost clear and contained besides most numerous cholera vibrios only few leucocytes and small numbers of erythrocytes

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If smaller doses were given, the peritoneal cavity as well as the blood and the intestines were found to be sterile.

Older rabbits proved to be rather resistant to intraperitoneal cholera infection, unless large-sized inocula were used.

It is of interest to note in this connexion that in the experience of Bezzola (1912) the resistance of rabbits to intraperitoneal infection could be over come by the administration of bile together with otherwise non-lethal doses of *V cholerae*

Sanarelli (1919a, 1919b) again drawing attention to the problems of intraperitoneal cholera infection, stated that in guinea pigs inoculated in this manner a rapid invasion of the blood-stream took place, regardless of whether non-lethal or lethal doses were used. In the case of non lethal infections, leucocytes agglomerating on the omentum acted as a potent barrier against further invasions of *V cholerae* phagocytizing the organisms and becoming ultimately ingested in their turn by large mononuclear cells. Vibrionaemia usually abated 2 3 hours after inoculation, and after 12 hours the blood was invariably sterile.

In the case of a lethal infection, phagocytic action was at first incomplete and a tardy revival of the leucocytic activity combined with the bactericidal action of the peritoneal fluid was incapable of checking the progress of the infection, though bringing about the sterility of the peritoneal cavity met with at autopsy Sanarelli maintained, therefore, that the intraperitoneally cholera infected guinea pigs did not succumb to peritonitis—a postulation which as will be gathered from the findings of earlier observers recorded above, was by no means novel However in contrast to these workers he insisted in another paper (1919b) that *V cholerae* exerted a toxic action not on the nervous centres but upon the intestinal tract, where an *acute and* fatal gastro-enteritis resulted. Such an evolution was possible because in Sanarelli s opinion the blood of guinea pigs did not exert a bactericidal action upon the cholera vibrios. Consequently the organisms, passing from the peritoneum into the blood, multiplied there and were subsequently excreted through the intestinal walls. Moreover reaching also the stomach walls, they produced there functional and anatomical changes which were instrumental in rendering the stomach contents alkaline and thus facilitating an abundant multiplication of *V cholerae* within the stomach.

In a further article, Sanarelli (1920b) besides reiterating his previous statements claimed that an exturpation of the omentum, the principal centre of defence against the intraperitoneally injected cholera vibrios, rendered the invasion of the blood-stream by the organisms more rapid and copious, and consequently aggravated the "vibrio gastro-enteritis of peritoneal origin" He further alluded to the possibility that

"on account of their own motility the vibrios might even pass directly from the peritoneal cavity onto the intestinal wall via the network of lymphatics, which creates a communication between the subserosa and the submucosa" [Trans.]

Experimenting with rabbits as well as with guinea pigs, Sanarelli (1923a) found that in the former as well as in the latter the intraperitoneally injected cholera vibrios rapidly passed into the circulation and thence into the intestinal wall. He stated in this connexion that excretion of the vibrios by the rabbit intestine began as soon as a few minutes after inoculation and, though as a rule ceasing after 12 hours, could continue for a few days

Continuing his studies, Sanarelli (1923b) reached the conclusion that by intravenous injection of *E. coli* 6 to 7 hours after intraperitoneal cholera infection one could produce in these animals a condition analogous to the algid stage of human cholera. Further reference to this contention which was supported by Sdrodowski & Brenn (1925) will be made in a later part of this chapter

Observations confirming that after intraperitoneal inoculation of guinea pigs the cholera vibrios rapidly passed into the intestines were recorded by Masaki (1922) and Ray (1927) while Suzuki (1926) obtained identical results with an organism showing the properties of *V El Tor*

Intraperitoneal inoculation of white mice with *V cholerae* already resorted to by Koch (1884) and a few other early experimenters, was again amply used by modern workers such as Koesoemadilaga (1939) Griffitts (1942) Ranta & Dolman (1943) and more recently also by Gallut & Jude (1954 see also Jude & Gallut, 1955) and Husain & Burrows (1956) to whose observations attention has already been drawn when dealing with the problem of cholera immunology in Chapter 4

Koesoemadilaga (1939) found that intraperitoneal administration of cholera vibrios led to an invasion of the blood-stream within 10 minutes, whereas the invariably occurring invasion of the intestinal tract took place later than that of the blood. The organisms disappeared from the circulation 6-10 hours after infection.

As mentioned in the fourth chapter Griffitts (1942) demonstrated the possibility of infecting white mice by the intraperitoneal route with small doses of mucin-suspended cholera vibrios

Ranta & Dolman (1943) who resorted to intraperitoneal inoculation of white mice in the course of their work with cholera vaccines thus adequately described their findings

" Mice that succumbed to an intraperitoneal injection of *V cholerae* had fairly characteristic pathological signs. Within half an hour of the injection they hunched together in their cages, inactive and obviously ill. Death usually occurred within 16 to 24 hours, but where the dosage was barely lethal they often survived until the second or third day Autopsy findings revealed injected vessels in the skin and in the mesentery Invariably the upper nine inches of the small bowel, or slightly over half its length, was filled with a pale, greenish-brown viscous fluid, having a soft, jelly-like consistency In mice dying more than 48 hours after the injection, this fluid was dark green in colour The liver spleen, lungs, kidneys, brain and heart showed no typical features."

Cultures taken at autopsy, of more than 40 mice were found to be positive for *V cholerae* in the following percentages

Peritoneal cavity	100 0
Heart blood	100.0
Upper intestinal contents	88 0
Stomach contents	21 0
Anal contents	Never positive

Commenting on these findings, Ranta & Dolman pointed out that

"The finding of the vibrios in only 88 per cent of the upper intestines does not necessarily give a complete picture of the situation there, as all of those specimens reported negative were overgrown by spreader types"

Intrapleural inoculation

The method of intrapleurally inoculating experimental animals with *V cholerae* seems to have been utilized by a few workers only first apparently by Vincenzi (1892) who stated that guinea pigs injected in this manner succumbed to the infection in 20-30 hours.

Sluyts (1893) recorded that rabbits infected with cholera vibrios by the intrapleural route died within a few hours and showed at autopsy large amounts of the organisms in the blood as well as in the pleural cavity

In the experience of Kolle (1894) guinea pigs intrapleurally infected with *V cholerae* died after 6 hours to about 12 hours. At autopsy vibrios were plentiful in the chest cavity and invariably present in the blood where, however they were usually scanty In the case of two animals, which showed fair amounts of cholera vibrios in their blood the organisms were also scantily present in the intestine

Schoebl (1916a) was able to produce infection in but one out of 3 guinea pigs inoculated with *V cholerae* by the intrapleural route This animal, killed in a moribund condition 24 hours after injection, yielded positive peptone water cultures from the chest cavity as well as from the blood, peritoneum, lungs, spleen, and liver

Intrameningeal inoculation

The method of cholera inoculation by the intrameningeal route seems to have been used only by Urbain (1929) Though working with a culture of rather low virulence, he was able to produce in rabbits a rapidly fatal infection by injecting $\frac{1}{8000}$ $\frac{1}{10000}$ of an agar slant of this strain through the atlanto-occipital ligament. The animals died in 24 hours and showed at autopsy congestion of the brain, the medulla oblongata, and the spinal cord. Pure cholera cultures could be isolated from these parts as well as from the heart blood

Still lesser doses of the weakly virulent cholera strain used by Urbain produced in rabbits signs of a meningo-encephalitis. The animals thus affected died after 20-30 days in a cachectic condition

Intravenous Infection

While, in order to introduce cholera vibrios directly into the blood stream, numerous experimentators took advantage of the usual method of intravenous injection a few resorted instead to intra-arterial (intracarotic) or intracardial inoculations. Since, however, the results obtained by the last mentioned workers were not fundamentally different from those produced through intravenous introduction of *V cholerae*, it is legitimate to deal with them in the course of the present disquisition

As quoted by Emmerich (1885) and by Thomas (1893) the method of producing cholera in experimental animals through intravenous injections which as stated above (page 398) proved unsatisfactory in the hands of Koch (1884), had been used before discovery of the cholera vibrio Magendie (1832) in particular had been able to produce in dogs a syndrome similar to human cholera through intravenous injection of large amounts of blood withdrawn from cholera patients. At autopsy of the rapidly succumbing animals, changes more or less resembling those of human cholera were noted in the intestines. It is important to note that the validity of Magendie s observations has been confirmed through experiences with dogs which had been intravenously infected with pure cultures of *V cholerae* (Gamaleia, 1892 Klemperer 1894)

Though giving no details in this respect, Emmerich (1885) evidently resorted to intravenous infection of some of the animals (guinea pigs, cats, and dogs) used by him for cholera experiments for he claimed to have obtained proof

> that regardless of the mode of inoculating experimental animals (injection into the veins, the lungs, the peritoneum, or under the skin) the pathogenic organisms [*Pilze*] are able to penetrate sooner or later through the intestinal wall into the lumen of the intestine and to produce there severe changes " [Trans.]

Since, like Buchner (1885) Emmerich worked with a Neapolitan strain, the nature of which is uncertain the validity of his claims is doubtful

In the course of a carefully conducted study on " the fate of micro-organisms injected into the blood of warm-blooded animals " Wyssokowitsch (1886) used, *inter alia* cholera vibrios and Finkler & Prior s cholera like vibrios for the intravenous infection of rabbits He found that these organisms rapidly disappeared from the blood-stream, not because they were excreted into the intestinal or the urinary tract, but because they were retained in the internal organs. On account of these findings and of ana logous experiences with numerous other bacterial species Wyssokowitsch postulated that this retention was the mechanism at work in rendering the blood stream free from invading bacteria.

In agreement with this contention Tizzoni & Cattani (1888) found it impossible to infect normal guinea pigs through injection of cholera vibrios into the bared jugular vein. However, the two workers were able to produce

a rapidly fatal infection in 5 out of 7 guinea pigs which had received an intraperitoneal injection of opium tincture simultaneously with a cholera inoculum given intravenously The causative organisms were occasionally found in the blood of these 5 animals up to 20 hours after infection and were usually demonstrated in the peritoneal cavity and the internal organs. However with one rather doubtful exception, they were found to be absent from the intestinal contents of the infected animals.

In strict contrast to the observations recorded above Thomas (1893) found that intravenous injection of rabbits with adequately large doses of *V cholerae* led invariably, and usually within 18-36 hours, to a fatal infection, which was characterized by signs and symptoms resembling those of human cholera as well as by the presence of the causative organisms in the intestines in almost pure or even in pure culture

Making further parallel observations on normal rabbits and on other animals, into the common bile duct of which a canula had been introduced after double ligature Thomas established that, regardless of whether or not this operation had been made, the cholera vibrios invariably appeared in the bile of rabbits which had been infected intravenously with a lethal dose. Since the causative organisms could be always cultivated from the intestinal contents of the operated as well as the normal animals, Thomas was led to assume that

"the bacilli can reach the intestinal contents through the bile as well as directly through the intestinal walls."

A further observation of Thomas's was that oral administration of 20% 25% ethanol for 2 days previous to intravenous inoculation rendered the rabbits in question susceptible to infection with otherwise sublethal doses of *V cholerae*

In the experience of Diatroptoff (1894) intravenous injection of rabbits with cholera vibrios led almost invariably to an appearance of the organisms in the intestinal contents, regardless of whether the animals had succumbed after 1 2 days or whether they had survived for 5-6 days because they had received smaller doses or inocula of lower virulence However only the rapidly succumbing rabbits yielded positive cultures from their blood and internal organs.

In marked contrast to the findings of Thomas and Diatroptoff, Pfeiffer (1894) maintained that

"if one injects cholera vibrios into the blood-stream of normal guinea-pigs, particularly into the carotis, one finds that after but a few minutes a quite overwhelming part of the vibrios has been destroyed This rapid death of the vibrios is associated with most rapidly appearing signs of intoxication, which are quite identical with those appearing after intraperitoneal inoculation of highly immune guinea-pigs. On account of these findings it is easy to understand that in the case of intravascular injection into guinea-pigs the lethal dose of living bacteria is but slightly lower than that of chloroform-killed cultures." [Trans.]

Kolle (1894) furnishing details of the 7 experiments referred to by Pfeiffer summarized that

"even after introduction of very large doses, exceeding the minimal lethal dose ($\frac{1}{2}$ loop) 4-8 times, one found in these animals a few hours after injection no more living vibrios in the blood and the organs. Vibrios were totally absent from the intestines in 4 animals and were quite scanty in three." [Trans.]

In agreement with Pfeiffer's contentions Klemperer (1894) claimed on the basis of a few observations that intravenously infected rabbits succumbed to an action of the cholera toxin and not to an infection. It is noteworthy however that, while unable to cultivate cholera vibrios from the stools of his test rabbits, he found the organisms present or even numerous in the small intestines at autopsy of such animals.

More to the point than the rather sketchy observations of Klemperer were the results of systematic studies made by Issaeff & Kolle (1894) on 35 intravenously infected rabbits The conclusions arrived at by these two workers were as follows

"After injection of cholera vibrios into the blood-stream, rabbits fall sick and succumb under otherwise identical conditions the more certainly the younger they are. The animals which, in relation to their body weight, receive comparatively large doses die within 18 hours after injection with the signs of an acute intoxication. In this case the comma bacilli more or less abound in the blood and in the organs, [but] they are absent or quite scanty in the intestinal contents. If one has reached the lower limit of the dose, after the administration of which the animals die within a short time, one sometimes finds the blood sterile. One cannot, therefore, speak of a real vibrionic septicaemia. The gross and microscopic changes in the intestine as well as the diarrhoea present during life must be ascribed consequently to the action of toxins furnished by the cholera vibrios, which succumbed in the blood.

"Those animals which survive the first 18 hours after infection develop diarrhoea within the following days and mostly succumb after some time to an intestinal affection *completely analogous to human cholera.* In typical cases one finds in the contents of the intestine, which is congested and bereft of its epithelium, Koch's bacilli in pure culture. In most cases the organisms cannot be found in the organs and in the blood even with the aid of peptone water enrichment. The organs are macroscopically normal with exception of the liver which shows more or less fatty changes." [Trans.]

Baroni & Ceaparu (1912) repeated the work of Issaeff & Kolle but used in contrast to the latter exclusively adult rabbits for their experiments. Sacrificing their test animals at intervals varying from 5 minutes to 16 days after intravenous injection and making peptone water cultures from the organs as well as from various parts of the intestine they found that

(*a*) if large doses were given the vibrios appeared in the bile and the appendix after 30 minutes, but in the small intestines after one hour only even though a marked congestion of these intestines, accompanied by a watery exudation into the lumen, became manifest as little as 10 minutes after intravenous inoculation of *V cholerae*

a rapidly fatal infection in 5 out of 7 guinea pigs which had received an intraperitoneal injection of opium tincture simultaneously with a cholera inoculum given intravenously The causative organisms were occasionally found in the blood of these 5 animals up to 20 hours after infection and were usually demonstrated in the peritoneal cavity and the internal organs. However with one rather doubtful exception, they were found to be absent from the intestinal contents of the infected animals.

In strict contrast to the observations recorded above Thomas (1893) found that intravenous injection of rabbits with adequately large doses of *V cholerae* led invariably and usually within 18 36 hours, to a fatal infection which was characterized by signs and symptoms resembling those of human cholera as well as by the presence of the causative organisms in the intestines in almost pure or even in pure culture

Making further parallel observations on normal rabbits and on other animals, into the common bile duct of which a canula had been introduced after double ligature Thomas established that, regardless of whether or not this operation had been made, the cholera vibrios invariably appeared in the bile of rabbits which had been infected intravenously with a lethal dose. Since the causative organisms could be always cultivated from the intestinal contents of the operated as well as the normal animals, Thomas was led to assume that

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Nichols (1916) stated in an experimental study on the pathogenesis of gall-bladder infections in cholera as well as in typhoid and dysentery that

"In three rabbits given ear vein injections of 1 c.c. of a 24 hour [cholera] broth culture, I found no lesions and no vibrios in the bile at the end of 1 week. In three rabbits given the *same dose by a mesenteric vein* one animal had bloody bile but no vibrios were found in it."

However further observations by Nichols on rabbits, in which after ligature of the cystic duct a fistula of the common bile duct had been made and which were afterwards injected with 1 ml amounts of cholera broth cultures through a mesenteric vein, showed the organisms to be capable of passing into the first specimens of bile collected at short intervals within one hour after infection. Tests made in an analogous manner by the introduction of the vibrios into an ear vein gave less satisfactory results. On account of these findings, Nichols felt convinced that the cholera vibrios "regularly enter the bile from the liver if they are present in the blood in sufficient numbers" and suggested that, besides a general septicaemia, a septicaemia of the portal vein system might be of importance in the pathogenesis of cholera

Experimental studies continuing the work of Nichols were made by Mashimo (1923) and more recently by Bifulco (1932a 1932b 1948) The former observer intravenously injecting rabbits with cholera vibrios after a common bile duct fistula had been made confirmed that the organisms rapidly appeared in the bile demonstrable there in small numbers as early as 2 minutes after infection they soon became abundant and continued to be numerous up to the end of the observation period of 24 hours.

Bifulco who like Nichols resorted to inoculation of *V cholerae* into the mesenteric vein of rabbits and also of dogs, stated that he had been able to produce in this manner typical signs of enteric cholera secondary to an invasion of the liver and the bile of the animals.

Evaluating the findings of Nichols and of Bifulco and the claim made by the former regarding the importance of a portal vein septicaemia in cholera, one must note that results analogous to theirs have been recorded by other workers when injecting *V cholerae* not into the mesenteric vein but into other blood vessels of their experimental animals.

Further to the observations by Tizzoni & Cattani (1888) and Thomas (1893) quoted above according to which the resistance of experimental animals to intravenous cholera infection could be lowered through opium or alcohol *administration* Golovanoff (1923) found that rabbits when orally given 10 ml of bile mixed with licorice powder 4 hours before intravenous cholera infection succumbed to otherwise non lethal doses.

Similarly Arnold & Shapiro (1930) established that intravenous administration of half a lethal dose of *V cholerae* if followed even after a considerable interval by an intraduodenal injection of an alkaline buffered phosphate solution produced diarrhoea and death The causative

(*b*) if lesser but still lethal doses were used, the vibrios disappeared 48 hours after infection even in animals the death of which occurred some days later

(*c*) the stomach contents, urine and stools of the infected animals never yielded positive cultures.

In contrast to the observations just mentioned Violle (1912) found that adult rabbits which rapidly succumbed to an intravenous injection of cholera vibrios showed no lesions In animals succumbing with some delay the small intestines were intensely congested and had diarrhoeic contents. Violle added in a further publication (1914b) to which reference has already been made above (pages 414 and 415) that in adult rabbits intravenously injected with a lethal dose of a cholera broth culture, the organisms were present not only in the blood, but also in the contents of the small intestine The congestion of its mucosa was more pronounced in the lower part of the small intestine than in the upper part above the opening of the pancreatic duct. Correspondingly smears made from the upper part of the small intestine showed only scanty cholera vibrios, while the organisms abounded in the material collected below the opening of the pancreatic duct.

Again working with young rabbits, Cano (1913) found that intravenous injection of 2 ml amounts of cholera broth cultures led in 6 animals invariably to an appearance of the organisms in the intestinal contents. The presence of cholera vibrios was also demonstrated five times in the kidneys as well as in some instances in the urinary bladder the urine, or both

Following up an investigation on gall bladder lesions in human cholera victims, Greig (1914b) intravenously injected 10 adult rabbits with cholera vibrios Though the animals invariably became seriously ill after inoculation and some developed diarrhoea only 2 spontaneously succumbed to the infection However positive bacteriological findings were also made at autopsy of 3 rabbits which had been sacrificed The causative organism could be isolated not only from the bile but also from the intestinal contents or the heart blood, or both, of the five animals showing evidence of cholera infection. Histological examination resorted to in the case of one of the animals revealed the presence of gall bladder alterations which closely resembled those met with in human cholera

Greig also found that intravenous inoculation of rabbits with cholera like vibrios produced a generalized infection He afterwards showed (see Greig 1915 1916) that intravenous injection of rabbits with cholera like vibrios, if repeatedly administered over long periods, almost always produced a cholecystitis and quite often also led to a formation of gall-stones in the animals concerned. Almost without exception the cholera like vibrios continued to be present in the bile of the inoculated rabbits.

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organisms could be cultivated from the faeces of these animals during life, from their intestinal tract and internal organs at autopsy

To support his postulation that cholera in man was the result not of a direct gastro-intestinal infection but of a septicaemia engendered through an entry of the causative organisms through the faucial organs, Sanarelli laid great stress upon observations he made in experimental animals infected by the intravenous route

Dealing with this mode of infection in his study on cholera in young rabbits, Sanarelli (1921) pointed out that, when making injections into the jugular vein of such animals, it was necessary to use very small doses in order to avoid a rapid death due to a vibrionic septicaemia. If however such a generalization of the infection could be avoided an entry of the cholera vibrios through the blood as well as by other routes led to their elective localization in the digestive tube, where, regardless of which mode of infection had been chosen identical characteristic lesions were produced.

As stated by Sanarelli in his 1924 study on experimental cholera, in contrast to the young animals

"adult rabbits better resist the intravenous administration of cholera vibrios. The minimal lethal doses are those which kill after more than 48 hours. In these cases the anatomic picture is that of an enteritis and the bacteriological findings—as in guinea-pigs succumbing several days after an intraperitoneal injection of the vibrios—are characterized by sterility of the blood and organs, and by the constant presence of the organisms throughout the intestinal tract." [Trans.]

Sanarelli noted in this connexion that the cholera vibrios which had been intravenously administered to adult rabbits in non-lethal doses almost completely disappeared from the blood after 24 hours, but then began to appear in the ileum 48 hours after infection the blood was as a rule sterile, while the organisms multiplied in the intestinal tract, where they usually persisted for 5-6 days. However he maintained that

"sometimes even intravenous injection of the vibrios in a usually non-lethal dose is capable of suddenly killing the rabbits. In such cases death is caused either by a sudden exaltation of the virulence of other microbial species (generally colon bacilli) followed by their rapid invasion, or by an anaphylactic crisis provoked through the existence of old sensitizing microbial foci in the liver the lungs, etc., upon which the vibrios act as releasing ('déchaînants') antigens." [Trans.]

Sanarelli claimed in this connexion that, while intravenous injections of killed cholera cultures did not exert the sensitizing action described above an anaphylactic crisis could be provoked through intravenous administration of small quantities of coli toxin or proteotoxin. However while it is conceivable that such unspecific agents exerted an action through lowering the resistance of the test animals to infection with *V cholerae* one cannot share Sanarelli's belief in the universal importance of this factor and its role in the pathogenesis of human cholera.

Masaki (1922) also held that intravenous injection of cholera vibrios led to signs and to a distribution of the organisms identical with those resulting from subcutaneous or intraperitoneal infection As can be gathered from the single protocol he quoted in this respect, a young rabbit, which died 10 hours after intravenous inoculation of half a slant of a cholera culture, showed at autopsy marked congestion of the intestinal tract, particularly of the small intestines, which were filled with diarrhoeic contents Cholera vibrios were scanty in the blood and absent from the bile, the urine and the stomach but abounded in the intestines

Paralleling the observations of Sanarelli and of Masaki, Ray (1927) found that intracardial injection of cholera vibrios into guinea pigs led to a septicaemia and to the appearance of the organisms in the peritoneum the liver and the bile as well as in the intestines. Commenting upon these findings and analogous experiences with subcutaneous or intraperitoneal infection, Ray stated that

" the appearance of the vibrios in the intestine stands apparently in close connexion with their appearance in the blood. In the majority of cases, in which the vibrios were demonstrated in the blood, they could be found as well in the intestines Possibly therefore, the so-called enterotropism of the cholera vibrio could be explained by the assumption that the organisms reach the intestine as well as other organs through the blood circulation and find in the intestine particularly favourable conditions for their multiplication." [Trans.]

There can be no doubt that the explanation tentatively offered by Ray to account for the supposed enterotropism of *V cholerae* is fully valid without being in any way actively attracted by the intestinal tract, those organisms which have been passively transported there find in the intestines incomparably favourable conditions for their entrenchment and multiplication and are therefore able to produce an enteric affection, even though they have been administered by parenteral routes.

General considerations

As will be gathered from the evidence quoted above the views arrived at by the various workers regarding the nature of the processes engendered by parenteral cholera infection in experimental animals varied widely While some felt convinced that only intoxication resulted, others thought that a generalization of the infection occurred or even held that after a passing phase of bacteraemia parenteral cholera inoculation led in an obligatory manner to a purely enteric affection

However while there is no reason to doubt that the observations recorded by the advocates of these contending schools were as a rule authentic as far as they went, the workers concerned erred by ascribing a universal validity to the results of their individual investigations. For it is certain that depending upon the interaction of several variables among which— apart from the mode of infection chosen—the character of the strains

used the dosages employed, and differences in the susceptibility of the test animals were most important, parenteral cholera inoculation could lead either to a rapidly evolving intoxication or to a generalized infection and its sequelae, or finally, quite often to a combination of both these processes. It ought to be clear therefore that, however interesting the experiences with parenteral infection of different test animals often were, they should not be used to support any narrowly conceived theories.

Action of Cholera Toxin

Animal experiments

As mentioned already in the first part of Chapter 3 and also occasionally alluded to in the course of the present disquisition some of the early workers, following up the pioneer investigations of Nicati & Rietsch (1884d) and of Cantani (1886) experimentally explored the pathogenicity of the cholera toxin. The evidence quoted in this respect may thus be summarized and supplemented

Tizzoni & Cattani (1888) experimented not only with living cholera vibrios, but also with cultures of these organisms which had been killed by heating at 80°C for one hour They were able in this manner to cause death in (*a*) 2 guinea-pigs given orally 10 ml of a heat-killed broth culture after alkalinization of their stomach and also receiving opium tincture intraperitoneally (*b*) 7 out of 9 guinea-pigs given the toxin subcutaneously and opium tincture intraperitoneally (*c*) one out of 3 animals given ethanol intragastrically together with a subcutaneous toxin dose and finally (*d*) 3 out of 4 guinea-pigs receiving the toxin intraperitoneally and opium tincture subcutaneously

Noting that the animals successfully inoculated with toxin showed during life as well as at autopsy signs identical with those met with in cholera-infected guinea-pigs, Tizzoni & Cattani emphasized that regardless of the route of administration of either living or heat-killed vibrios " the morbid picture as well as the anatomical lesions are determined ultimately by the action of the cholera poisons "

As already referred to in Chapter 3 Pfeiffer (1892) fully confirmed these views by showing that it was possible to kill guinea-pigs through intraperitoneal injection of either killed or living cholera vibrios, provided that in the former case adequately increased doses of the organisms were used, since they were no longer capable of a preliminary multiplication in the animal body

Pfeiffer established in this connexion that admixture of chloroform or thymol to broth cultures of *V cholerae* or finally drying of thin layers of agar-grown vibrios in glass dishes at 37°C for 24 hours were the best methods to obtain the toxic materials. Having had bad experiences with exposure of his cultures to higher temperatures (60°C or 100°C) Pfeiffer was led to assume that through this procedure the " primary " cholera toxin was changed into a less acutely active secondary modification. He also concluded from a few experiments with glycerin broth cultures and with suspensions of *V cholerae* in glycerin and then in broth, that Chamberland-filtrates of cholera cultures exerted no, or at least no lethal, toxic action.

Sobernheim (1893) while otherwise confirming Pfeiffer's experiences with intraperitoneal administration of cholera toxin, established in agreement with the observations of most other workers that the filtrates of cholera broth cultures exerted a typical toxic action, provided that growths of a sufficient age (in Sobernheim's experience 30-day-old cultures) were used for experimentation. He concluded, therefore, with much reason

that the toxicity of the filtrates was due to " the death and disintegration [*Auslaugung*] of a great number of bacterial bodies "

Sobernheim s experiences with oral administration of cholera toxin have already been recorded above (see page 399).

Experimenting with agar-grown cholera vibrios killed with the aid of chloroform vapours, Issaeff & Kolle (1894) established that this material, while highly toxic for intravenously injected guinea-pigs, was far less active for rabbits inoculated by the same route whereas $\frac{1}{4}-\frac{1}{2}$ loop of the killed growths sufficed to kill the former animals within a few hours in a typical manner the certainly lethal dose for intravenously injected rabbits varied from $1\frac{1}{2}$ to $2\frac{1}{2}$ loops. Moreover these animals succumbed to the toxin much less rapidly than the guinea pigs, even an animal which had received the enormous dose of 60 loops into the ear vein dying only after 2 days. It is interesting that the toxin-inoculated rabbits, while not manifesting marked oscillations of their temperature, suffered as a rule from diarrhoea. At autopsy they invariably showed fatty changes in their liver

There is no need to adduce at the present juncture the experimental evidence regarding the relations existing between the toxicity and the virulence of *V cholerae* because this subject has already received attention when the problems of immunology were discussed in Chapter 4

As also stated there, Ransom (1895) worked with a soluble toxin obtained by heating 5- to 10-day-old cholera broth cultures of *V cholerae* for a short time at 100°C and passing the fluids through Pukal filters, and with a solid toxin prepared from the filtrates. Details referring to the action exhibited by these two toxins in experimental animals were as follows

0.5-ml doses of the fluid toxin were sufficient to kill either intraperitoneally or subcutaneously inoculated guinea pigs within 24 hours. Administration of lethal doses invariably produced marked signs of collapse. At autopsy of the subcutaneously injected animals no or no marked, signs were found at the site of the inoculation. There was a considerable amount of sometimes slightly blood-stained fluid in the peritoneal cavity The intestine and the suprarenals were congested.

The soluble toxin was also lethal for rabbits, a dose of 4 ml given subcutaneously sufficing to kill animals of 1500 g within 24 hours. White mice and pigeons appeared to be insusceptible.

Parenteral administration of the solid toxin was effective in much smaller doses, 0 07 g representing the minimal lethal dose for subcutaneously injected guinea-pigs. Multiples of this dose, which usually killed the animals after 6-8 hours, caused death within a very short time. It was not possible to infect guinea-pigs through the administration of even large doses of the solid toxin with their food.

The endotoxin which, as stated in Chapter 4 Schurupow (1909) obtained through alkali treatment of *V cholerae* cultures followed by candle filtration, exerted in intraperitoneally inoculated guinea pigs an action similar to that of Ransom s soluble toxin and also marked signs of intoxication in horses. Some of these animals, injected intravenously with 5-10 ml doses of the endotoxin, i.e., apparently with amounts gathered from 1 3 agar slants, succumbed within a few days or even after 2 11 hours, after having shown signs of profound collapse and diarrhoea

Discussing the effect of oral doses of cholera endotoxin, Freter (1955) drew attention to the work of Bürgers (1910), who was unable to kill guinea-pigs by feeding them with large amounts of heat killed cholera vibrios—a result confirmed by Freter in the case both of these experimental animals and of mice.

Turning attention to later and recent observations in regard to the action exerted by cholera toxins on experimental animals, reference has to

be made first to the work of de Bonis & Natale (1913) As already noted in the fourth chapter they found that intragastric administration of cholera nucleoproteid (1 3 doses of 0 005 g to 0 02 g dissolved in 0.5% sodium bicarbonate solution) exerted a lethal effect upon 9 out of their 11 test guinea pigs Given intraperitoneally, the nucleoproteid was lethal for 250-g guinea pigs in 1 mg doses.

Cicconardi (1914) also working with the cholera nucleoproteid of Galeotti (1912) found this product to be lethal for rabbits in doses of a few milligrams death occurring rapidly after intravenous injection, more slowly after intraperitoneal inoculation. Guinea pigs were, in the experience of this worker less amenable to the action of the nucleoproteid.

As further stated by Cicconardi, administration of the cholera nucleoproteid, besides causing a marked fall of the blood pressure and a slowing of the pulse in rabbits, produced (*a*) respiratory disturbances (hurried and laboured breathing or different types of irregular breathing) apt to terminate in respiratory paralysis (*b*) an increased peristalsis of the gut and (*c*) suppression of the urine which did not seem to be secondary to an action of the toxin on the kidney vessels A slight increase of the red blood corpuscles appeared to be the result of peripheral stasis. Leucocytosis was occasionally observed.

The experiences of Puntoni (1913) with daily oral administration of cholera toxin together with sufficient doses of sodium carbonate solution to produce slight abrasions of the gastro-intestinal mucosa suggested that the toxin was more potent in a series of guinea pigs kept in a warm and humid atmosphere (30° 32 C with 90% 95% saturation of the air) than in a series kept at 17 C to 19 C with 50%-60% saturation of the air It is however important to note that recent observations of Gallut and Jude referred to in Chapter 4 were not in accord with the postulations of Puntoni. Thus, as was mentioned in that chapter Gallut & Jude (1954) stated that, depending upon variations in the toxigenic power of their cultures, the virulence of the growths incubated at 18 C was highest, and that of the vibrios grown at 41 5 C lowest. Further Gallut & Jude (1955) established in accordance with these findings that the most active cholera toxins were obtained from cultures kept at 18 20°C, the comparatively most feeble from growths incubated at 41 C while the toxicity produced at 37°C was of an intermediate degree

Interesting observations of Penfold & Violle (1914) showed that intravenous injection of rabbits with distilled water at the rate of 1/30th of the body weight, while innocuous *per se* greatly enhanced the toxicity of otherwise sublethal doses of either unfiltered or filtered cholera broth cultures administered simultaneously The same result was obtained when intraperitoneal inoculation of sublethal toxin doses was followed 2 hours afterwards by intravenous injection of distilled water Since the injection of lysed blood in small quantities together with sublethal doses of cholera

cultures also produced rapid death in rabbits, Penfold & Violle reached the conclusion that the action of distilled water injections described above depended largely upon a lysis of the erythrocytes. The phenomenon was not specific for *V cholerae* or its toxin, because identical results were obtained with cultures of some other micro-organisms

The conclusions reached by Démétrescu (1915), when examining the suprarenals of rabbits subcutaneously injected with heat killed suspensions of *V cholerae* were that

(1) The cholera endotoxin produced in most of the 9 test animals an almost complete disappearance of the chromaffine substance as manifested by the disappearance of the normal colour reactions.

(2) Extracts prepared from the suprarenals of the inoculated animals contained no or almost no adrenaline because

(*a*) they produced only a slight or even no elevation of the arterial pressure

(*b*) they failed to produce the Ehrmann-Meltzer reaction (mydriasis) in the eyes of frogs and

(*c*) they did not give the characteristic colour reactions with phosphomolybdenic acid.[1]

Sanarelli (1920a) in order to study the toxic action of *V cholerae* prepared a "cholera proteid" by emulsifying the material from 24-hour agar cultures in a 0 1% solution of sodium carbonate and adding to each 9 ml of this emulsion 1 ml of a 1% solution of pancreatin and 4-5 drops of toluene After vigorous shaking, the tubes containing these mixtures were incubated at 37 C. On the following day the tubes were found to contain a slightly opaline but fully transparent fluid, because the pancreatin had been capable of dissolving the vibrios killed by the toluene

Experimenting with guinea pigs, Sanarelli found that these animals were not affected by subcutaneous injection of even large doses of the cholera proteid In regard to intraperitoneal inoculation he maintained that

"the peritoneal absorption of the proteid as well as of simply heat-killed vibrio cultures takes place in the guinea-pigs in an imperfect manner It follows that the minimal lethal doses of killed cultures introduced by the peritoneal route must always be a multiple of the minimal lethal dose of living cultures." [Trans.]

Intravenous injection of guinea pigs with the cholera proteid, on the contrary was lethal with dosages corresponding to those of the living vibrios. As stressed by Sanarelli, the toxic action of cholera proteid administered by this route

"was not exerted directly on the nerve centres, as certain authors had postulated, but [it] reaches *a tergo* the mucosa of the digestive tube where it produces an acute and fatal gastro-enteritis" [Trans.]

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[1] In connexion with these observations, it is interesting to note that according to Galhet (1955) extirpation of the suprarenals markedly enhanced the susceptibility of white mice to the action of cholera endotoxin.

Referring to his experiences with intraperitoneal and intravenous inoculation of rabbits, Sanarelli summarized that in these animals

"even if injected intraperitoneally the toxic action of the proteid extracted from the cholera vibrios is exerted electively on the digestive canal, producing, according to the dosages used, a fatal acute, subacute or chronic gastro-enteritis. These affections of the walls, characterized by very grave anatomical and functional lesions, are constantly accompanied by a most massive multiplication of *B coli* in the intestine, and can terminate in a general bacillary infection." [Trans.]

Acton & Chopra (1924) studied the toxic action of the protein bases of *V cholerae* isolated with the aid of the following method

A simplified Martin's broth was prepared by mincing veal finely soaking it for 24 hours at 37°C, then boiling, filtering, and neutralizing this digest and adding 0.5 / peptone and sodium chloride to it.

Cholera vibrios were grown in this broth for 10 days at 37°C with occasional shaking to allow free access of air to the medium. After 10 days cultivation the growth was heated for 5 minutes at 100°C. After cooling, the culture was filtered through paper and phosphotungstic acid was added to the filtrate to obtain a precipitate. The clear fluid obtained after addition of acetone to the precipitate and filtration was treated with baryta water to get rid of the phosphotungstic acid. After filtration the barium was precipitated by blowing CO_2 through the solution and a little weak sulphuric acid was used to remove remaining traces of barium. The resulting fluid was then concentrated over a water-bath, the concentrated extract was filtered, neutralized with weak Na_2CO_3, and made faintly alkaline. The extract was finally heated over a sand bath at 100°C to distil over the volatile protein bases.

Acton & Chopra established that the cholera toxin was contained in the non-volatile protein bases which remained in solution. While capable of making some pharmacological tests with this toxin, they had only 150 mg of the material left to inject 2 young rabbits. One of these animals, receiving 50 mg, showed no characteristic signs except slight cramp of the hinder limbs, and recovered. The second rabbit, which was injected with 100 mg (route not stated) soon collapsed and was killed after 1 hour At autopsy the small intestines were found to be markedly congested, while the peritoneal surfaces had lost their shiny appearance owing to a slight exudation of lymph. Histologically some vascular engorgement, cloudy swelling of the secreting tubules, and intratubular oedema were found in the kidneys.

Hahn & Hirsch (1926) claimed to have obtained a cholera toxin through cultivation of the organisms for only 6-10 hours in broth containing small amounts of glucose and kept at a constant pH of 8 0 They found that the centrifugate of such growths, sterilized with chloroform or toluene produced characteristic signs (collapse respiratory cramps and paralysis of the extremities) in guinea pigs injected intraperitoneally with 0.25- to 1 ml amounts and caused the death of these animals within 12 18 hours. At autopsy the suprarenals and often also the small intestines were found to be congested and exudates were seen in the serous cavities. Hahn & Hirsch also stated that the toxic action of the centrifugates was abolished

through heating at 70 C for half an hour and was markedly weakened by filtration through Berkefeld candles.

It is important to note that, as can be gathered from a subsequent elaborate publication of Hahn & Hirsch (1929) the explanation of their extraordinary findings lies in the fact that they worked almost exclusively, not with classical cholera vibrios, but with El Tor strains and even used for their early studies one strain which as convincingly shown by Andu & Niekerk (1929) and Soeleiman & Niekerk (1930) was immunologically different from *V cholerae* There can be no doubt, therefore that Hahn & Hirsch did not observe the action of the endotoxin of this organism, but worked with exotoxins mainly with the haemolysin of the *V El Tor* In fact, they admitted in their 1929 article that

"the toxin can be demonstrated in the first place in those strains which clearly produce a haemolysin on sheep blood agar plates. A relation between the haemolytic properties and the toxic function is rendered likely by the observation that the amount of [immune] serum neutralizing one lethal toxin dose also neutralizes the haemolytic effect correlated with this amount of toxin." [Trans.]

Ghosh (1933) claimed to have obtained a product which he considered to be an exotoxin of *V cholerae* by (*a*) cultivating the organisms under a layer of paraffin i e under anaerobic conditions, in a broth prepared with Martin s peptone and adjusted to a pH of 8 0 with sodium carbonate and (*b*) filtering the fluid after an incubation for 20 hours through L_3 candles. 5-6 ml of the filtrate caused the death of intravenously injected rabbits in $1\frac{1}{2}$ 2 hours a preliminary inoculation of 2.5 ml of the toxin, followed by further injection of 0 75-ml amounts 4 hours later and again the next morning led to the appearance of diarrhoea, which could be maintained by 2 daily injections of 0.5 ml until the animals died of uraemia. Ghosh maintained in this connexion that

"the cholera toxin exerts a specific action on the secretory glands of the small intestine, which produce a large quantity of alkaline stools, the characteristic rice-watery stools of cholera An irritating action is exerted on the kidneys. It is probable that the uraemia is caused through the elimination of large quantities of fluids and alkaline salts of the body and through the toxic effect on the kidneys." [Trans.]

Autopsy findings in the toxin injected rabbits varied according to the length of illness in the case of animals succumbing within a few hours the small intestine was but slightly congested In the animals succumbing 2-4 days after inoculation this congestion was marked and petechiae were present in the small intestine

Working with a cholera endotoxin prepared from broth cultures according to the method of Besredka & Golovanoff (1923 see Chapter 4) Pham (1935) found these filtrates inert when subcutaneously administered to guinea pigs, while a dosage of 0 8 ml was necessary to cause death through intracardial inoculation However spectacular results could

be obtained through injection of much smaller doses into the vicinity of the splanchnic nerve guinea pigs which had received 0 1 ml amounts by this route showed after as little as 1 hour shock, hypothermia, abdominal distension, and anuria, and died after 4 hours At autopsy, the terminal portion of the small intestine showed a haemorrhagic infiltration, while the plaques of Peyer were found to be congested The intestinal lumen was filled with fluid containing whitish flocculi

Doses of 0 05 ml, injected in the manner described above produced death in guinea pigs after 24 hours, while the animals receiving $\frac{1}{50}$ ml survived for 3-4 days, showing diarrhoea, oliguria with albuminuria, and a marked loss of weight. Rabbits given 2 ml injections into the vicinity of the splanchnic nerve showed identical signs, and died after 24 hours.

Histologically marked epithelial desquamation was found to be present in the small intestines the capillaries of their submucosa showed a considerable distension and were in part ruptured. Alterations in the kidneys concerned mainly the glomeruli which showed sometimes a hyaline degeneration, and sometimes oedema and haemorrhages Apart from some parenchymatous hepatitis and congestion of the suprarenals the other organs showed no abnormal findings.

Recently making further experimental studies on the action of cholera toxin De Sarkar & Tribedi (1951) resorted for this purpose to intraperitoneal injection of rabbits with vibrio suspensions killed through a 15 minutes exposure at 56 C. As these workers found the administration of the toxin led (*a*) to an outpouring of fluid into the peritoneal cavity and as a consequence to haemoconcentration, and (*b*) to a fall in blood pressure conditioned partly by the haemoconcentration and partly by a direct action of the toxin on the cardiovascular system

The appearance of the peritoneal effusion and of an oedema demonstrated histologically in the myocardium as well as in the mucosa and submucosa of the small intestine stood in causal connexion with an increased capillary permeability "brought about by a direct local action of the cholera toxin and also by its remote specific action on the capillaries of the myocardium and the inner coats of the small intestine"

As further stated by De and colleagues the cholera toxin

"also affects the vasculature and the parenchyma of the kidneys, leading to diversion of the toxin-laden blood from the cortex to the medulla. The vital cortical elements are thus protected from the effects of the toxin which, however damages those parts of the nephrons situated in the medulla and juxta-medullary regions. As the majority of the glomeruli are rendered ischaemic filtration of urine is diminished and further loss of body fluid by this route is prevented. This preventive mechanism fails when a very heavy dose of cholera toxin is injected."

It has to be noted that conclusions to some extent anticipating those of De and co-authors had been reached by Fujii (1924) through a study of the

kidney changes produced in guinea pigs through subcutaneous injection of small doses (1 mg per kg of body weight) of heat killed cholera vibrios As Fujii put it,

" In short, the nephrose of the urinary canal is brought about in the kidney of experimental cholera in from 8 to 24 hours. The swollen epithelia do not hinder the urinary stream but press the vas afferens and the vas deferens leading to the glomerules, thus compelling the blood of the canal of the Malpighian bodies to show either a state of anaemia or one of no blood at all. Then the action of diluting the urine in the glomerules decreases or ceases, while the urinary canal with nephrosis is incapable of concentration when the excretion of the kidney is entirely suspended."

While in view of the vastly divergent methods used by the numerous workers quoted above and the discrepant results recorded by some of them it is difficult to come to generally valid conclusions, the bulk of the available evidence leaves no room for doubt that the endotoxin of *V cholerae* is apt to produce in experimental animals signs and lesions closely resembling those which result from inoculation with comparable doses of living organisms. It is justifiable to postulate, therefore, that also if administration of the latter is resorted to the manifestations produced in experimental animals are largely caused by the toxin and not by a direct action of the cholera vibrios. This may be claimed to hold true even in those instances in which a generalized infection with *V cholerae* is demonstrable. In fact, one might almost say that in such animals the presence of the organisms in the blood and internal organs to a large extent camouflages the action of the cholera toxin instead of superseding it.

Since, as will be discussed in a later part of this chapter notwithstanding some claims to the contrary, a vibrionaemia is exceptional in man, it would seem at first glance that in human cholera more still than in the case of experimental animals the action of the endotoxin of *V cholerae* ought to be of paramount importance However as will be shown, this assumption appears to be justified to a lesser extent than is claimed by some authorities.

Tests with isolated organs

Tizzoni & Cattani (1888) evidently the first workers taking advantage of the method presently under review maintained that the organs of healthy experimental animals, kept *in vitro* at a temperature of 35 C in sterilized fluid cultures of *V cholerae* showed changes analogous to those in the corresponding tissues of cholera infected animals.

The action of cholera toxin on the isolated rabbit heart was studied by Cicconardi (1914) who ascribed the sudden slowing and the irregularity of the beat produced by the toxin to an interference with the nervous mechanism of the organ and not to a direct influence on the myocardium.

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For a further study of the action exerted by the cholera toxin on the isolated rabbit intestine Hahn & Hirsch utilized the toxic products prepared according to the procedure described by them in 1926 (see page 444) They found (Hahn & Hirsch, 1927) that these substances, if added in a low concentration (0 02%-0 0006%) to Tyrode solution caused a shortening of the contraction amplitude of the intestinal loops immersed in such fluids, which was apt to lead to complete paralysis. As established by the two workers in the course of further investigations (1928) perfusion of the intestinal loops with the toxic solution did not exert such an effect on the peristalsis. Hahn & Hirsch felt entitled to conclude from these observations that the cholera toxin was active only when it was administered parenterally However as has been discussed before (see page 445) this rather surprising conclusion as well as the postulations of these two workers in general cannot be considered as valid since the authors did not experiment with the endotoxins of true cholera vibrios, but utilized the soluble toxins of El Tor or even cholera like vibrios

Soeleman & Niekerk (1930) though finding like Andu & Niekerk (1929) that the toxin of a cholera like strain was capable of diminishing or even inhibiting the peristalsis of isolated pieces of the rabbit small intestine obtained entirely negative results in analogous tests with the toxin of true cholera strains They admitted, however that these failures might have been due to extrinsic causes, particularly an insufficient purification of their toxin the irritating action of the unspecific components of their products possibly masking their essential paralysing effect.

Though finding that within the limits of their experiments the cholera toxin did not influence the peristalsis of pieces of small intestine taken from freshly killed guinea pigs or rabbits, Burrows Wagner & Mather (1944) recorded the following important experiences (already shortly mentioned in the fourth chapter) with these and other living semipermeable membranes (pieces of frog skin)

"The rate of flow through the normal membrane immersed in Ringer Locke solution was found to be about 0.25 ml per hour The addition of living vibrios, or crude or purified toxin to the solution either within or outside the membrane markedly accelerated this rate The acceleration of flow began to be apparent at a concentration of 0.5 MLD (Mouse) per ml and increased with increased concentration of toxin until with 4 MLD per ml the rate was approximately double that of the controls With higher concentrations more rapid flow could be produced—we have observed as much as 6-fold differences —but the results were erratic. It was immaterial whether the toxin was placed inside or outside the membrane within or outside the lumen in the case of strips of intestine "

Additional tests with strips of intestine which had been soaked overnight in Ringer Locke solution containing 4 MLD of toxin per ml, but had been thoroughly washed before use gave results not differing from control tests with normal intestines thus furnishing further proof that the acceleration of flow described above took place only in the presence of the cholera toxin.

Further studies of the action of cholera toxin on the isolated heart of rabbits were made by Manwaring Boyd & Okami (1923) who resorted for this purpose to perfusion of the organ with well aerated Locke s solution containing 1% 2% of carefully filtered defibrinated rabbit blood and 5% 10% of Berkefeld filtrates of 2 to 7-day-old broth cultures of *V cholerae* It was found that these mixtures were almost non toxic for the conductory and contractile tissues of the excised mammalian heart, but markedly toxic for the capillary endothelium as evidenced by the appearance of myocardial oedema and diapedesis of erythrocytes, which led to the formation of numerous extravasates beneath the endocardium and pericardium

In contrast to these findings, Acton & Chopra (1924) stated that the non volatile toxic bases which they had isolated from cholera cultures (see above page 444) exerted, if used in a quantity of 2 mg for the perfusion of the isolated heart of a rabbit, a stimulating action on the organ They maintained therefore that the fall in blood pressure becoming manifest after intravenous injection of a cat with their toxic bases was the result of a dilatation of the vessels supplying the small intestines combined with an increased permeability of the endothelium of the vessels in the splanchnic area

Soeleiman & Niekerk (1930) experimented on the isolated heart of rabbits with a toxin which, following the technique used by Andu & Niekerk (1929) they had obtained by (*a*) centrifuging and evaporating 18-hour-old cholera broth cultures (*b*) precipitating the residue with 96% ethanol (*c*) pouring off the alcohol after 24 hours standing at room temperature and (*d*) evaporating the residue to complete dryness. For the perfusion tests solutions in distilled water were prepared, which contained 40 mg of the precipitate per ml

Soeleiman & Niekerk found that the toxins obtained in this manner from several true cholera strains did not exert an action on the isolated rabbit heart. They were on the contrary able to confirm previous findings of Rothberger (1905) and of Andu & Niekerk (1929) that the toxins of cholera like strains were capable of exerting such an action

Experimenting with the isolated uteri of guinea pigs, Acton & Chopra (1924) found that their non-volatile toxic bases produced even in a dilution of 1/250 000 histamin like contractions which became particularly marked in pregnant animals

Acton & Chopra, who also seem to have been the first to study the action of cholera toxin on the isolated intestines of experimental animals, noted in the case of rabbits

"an increase in the tone and amplitude of contractions when the non-volatile bases were tested in a dilution of 1-60 000 At a dilution of 1 120 000 the tone was slightly increased but the contractions were smaller and more irregular With volatile bases a more marked effect was seen."

or negative results regardless of the serological character of the vibrios in question, so that "in other words the pigeon pathogenicity method of classification does not agree with the serological" Greig refuted, therefore with much reason, the suggestion of Chalmers & Waterfield (1916) that tests with pigeons were useful for a distinction of different species of cholera like vibrios

Chick-embryos

Interesting observations on the fate of cholera and cholera-like vibrios in fertilized eggs were made by Wilson (1946) who used for this purpose 3 strains of *V cholerae* as well as 2 strains belonging respectively to the subgroups III and IV of Gardner & Venkatraman (1935) If 7-day-old chick-embryos were inoculated with any of these organisms via the allantoic sac, they died within 1 or 2 days except when such small doses were used that probably no vibrios were contained in the inoculum. Shortly before the death of the embryos the organisms could be cultivated not only from the allantoic fluid, but also from the amniotic fluid and the yolk

Frogs

As far as could be ascertained, David (1927) was the first worker who experimentally infected frogs with *V cholerae* presumably through injection into the dorsal lymph-sac. He recorded in this connexion that the animals which had received larger doses of classical cholera or El Tor vibrios cultivated at room temperature died after 4-8 days and showed signs identical with those met with in frogs infected with *V piscium*, a cholera like fish pathogenic vibrio studied by him (see below) It should be noted that David found in the latter animals an acute gastro-enteritis and haemorrhages in the internal organs There was reason to assume that the *V piscium* was pathogenic for frogs under natural conditions.

Using dosages of 200 million cholera vibrios, Gohar & Makkawi (1948) succeeded in infecting frogs of 20 g body weight by the intramuscular route Animals kept at 18°C died in 24 hours, those kept at 37°C in 12 hours. At autopsy the organs which were found to be congested, as well as the blood yielded positive cultures.

Frogs which had been injected intramuscularly with a toxic solution, obtained by treatment of cholera vibrios with NaOH and subsequent neutralization with hydrochloric acid succumbed in 24 hours.

Lizards

Goéré (1913) orally administered to 4 of the green lizards commonly met with in Tunisia 0 5-ml doses of a 24-hour broth culture of a human cholera strain. One of these animals developed acute diarrhoea with

PATHOGENICITY FOR LOWER ANIMALS

Pigeons

It is of historical interest to note that the question whether the cholera vibrio was pathogenic for pigeons formed the subject of a debate between Gamaleia (1888) and Pfeiffer & Nocht (1889). Gamaleia claimed that it was possible to enhance the virulence of *V. cholerae* through some passages through pigeons to such a degree that the organisms, if administered to these animals in minimal doses, produced a rapidly fatal generalized infection with localization in the intestines. Pfeiffer & Nocht stated on the contrary that (*a*) it was not possible to produce cholera in pigeons through oral or intravenous administration of the causative organisms, (*b*) "relatively enormous" doses were necessary to obtain positive results through intraperitoneal or intrapleural inoculation, and (*c*) no evidence could be adduced to show that passage through pigeons enhanced the virulence of the organisms. On the basis of these experiences and of an exhaustive study of the *V. metchnikovi* Pfeiffer (1889) reached the conclusion that the latter cholera-like vibrio

"was quite extraordinarily pathogenic for pigeons, whereas cholera [vibrios] possess practically no [*so gut wie gar keine*] virulence for this animal species" [Trans.]

Statements supporting the postulation of Gamaleia were made by a few other authors,[1] while Ray (1927) concluded from a few observations that the subcutaneous administration of large doses of cholera vibrios ($\frac{1}{6}$-1 slant) was apt to cause a fatal septicaemia in pigeons.

However, far more important than these claims is that, as established through tests with 65 strains by Kolle & Gotschlich (1903), intramuscular inoculation of pigeons with small doses of *V. cholerae* did not produce infection.

To judge from these consistently negative results it would seem at first glance that the method of intramuscularly inoculating pigeons with small doses of suspect strains would be of great value for the identification of true cholera vibrios. Unfortunately, however, as shown already by Kolle & Gotschlich and confirmed by Greig (1917), cholera-like vibrios do not uniformly produce positive results if tested in this manner. In fact, Kolle & Gotschlich found among their 22 cholera-like strains only 6 which, if introduced into the pectoral muscle of pigeons with the aid of a platinum-needle, i.e., in minimal amounts, produced a fatal vibrionaemia. Similarly Greig obtained with the same method only 8 positive results when testing 24 of his cholera-like strains, while 16 strains failed to cause a lethal infection even if administered in comparatively large doses. He stressed in this connexion that intramuscular inoculation of pigeons yielded either positive

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[1] See, for instance, Vincenzi (1892), Salus (1893) and the summaries of Sticker (1912), p. 262, and of Kolle & Prigge (1928), p. 41

Similarly Remlinger & Nouri (1908b) stated that they had isolated from oysters and mussels collected at Constantinople a vibrio which because it was agglutinated by a high titre cholera immune serum only in a dilution of 1/100 had to be considered as a cholera like organism The two workers stressed that such vibrios could be responsible for the appearance of choleriform diarrhoea in man

In two articles published in 1909 and 1912 respectively Bergman reported (1) on the isolation of a cholera like vibrio (*V anguillarum*) found to be responsible for an infectious tumour disease (*rote Beulenkrankheit*) of eels, and (*b*) on that of a similar or possibly identical organism which was the cause of an infective eye disease (keratomalacia) in codlings (*Gadus morrhua*) and, as shown by one observation, also of a gingivitis terminating in septicaemia in pike

The organisms in question were capable of liquefying gelatin and grew well in peptone water but differed in other respects from *V cholerae* particularly by not giving a cholera red reaction and growing best at room temperature

The vibrios isolated from the codlings proved to be experimentally pathogenic not only for this species for eels, carp and roaches, but also for subcutaneously infected crayfish, for 1 subcutaneously and 1 out of 2 intraperitoneally infected mice A few tests with guinea pigs (subcutaneous and intraperitoneal infection) and with intravenously or intraperitoneally inoculated rabbits gave negative results.

Though showing some differences in agglutination tests with sera raised in rabbits with the organisms isolated from codlings, the various strains studied by Bergman belonged obviously to one serological group of fish pathogenic vibrios.

Defressine & Cazeneuve (1914) demonstrated the presence of cholera like vibrios in 20% of the mussels collected in France at a short distance from the river Neuve which had been found to be contaminated with true cholera vibrios. The cholera like organisms though identical with *V cholerae* in regard to their morphology staining, cultural and fermentative properties and found to be pathogenic for guinea pigs and rabbits differed from classical cholera vibrios by their serological reactions, haemolytic power and inability to reduce nitrates to nitrites.

A further profound study of a cholera like vibrio responsible for violent and recrudescent epizootics among carp bred in a pond near Vienna Austria was made by David (1927) The organisms in question designated as *V piscium*, differed from cholera vibrios not only serologically but also in other respects.

Particularly noteworthy in the latter connexion is that they (*a*) failed to give the cholera red reaction (*b*) grew poorly in peptone water and (*c*) showed an aberrant behaviour when cultivated on agar their growth optimum on this medium ranged from 8 C to 20 C Involution forms

plentiful vibrios in the stools which contained whitish granules, and succumbed after 30 hours. A second lizard had diarrhoea for only 2 days, but continued to harbour the vibrios in its stools until it died in a cachectic condition after a month The other 2 animals showed only passing diarrhoea continuing to excrete the vibrios in their stools for 2 and 5 days respectively

Interesting as these observations are, it is difficult to share Goéré s belief that lizards could become carriers of *V cholerae* under natural conditions

Fish and shellfish

The problem of the natural occurrence and persistence of *V cholerae* in aquatic animals is of great practical importance because (*a*) as has been discussed in Chapter 3 this organism is apt to survive for considerable periods or even to multiply in fish and shellfish stored for consumption and (*b*) it has been repeatedly found that such contaminated foodstuffs have been the cause of cholera manifestations in man. It deserves attention in this respect that, as recorded by Takano Ohtsubo & Inouye (1926) a report from the sanitary authorities of the Kochi Prefecture in Japan stated

"that cholera vibrios were recovered from a large proportion of the fish caught in the bay which had been polluted with cholera."

More convincing than this information are the observations of Kundu & How (1938), who though as a rule finding only cholera like vibrios in the prawns, lobsters, and shrimps brought in baskets from the delta districts of Burma to the Rangoon market, were in two instances able to isolate *V cholerae* from prawns.

Before further dealing with the fate of cholera vibrios in fish and shell fish, it is important to refer to observations demonstrating the presence or even the pathogenicity of cholera like vibrios in these aquatic animals.

Klein (1905) studying the bacterial flora of oysters and mussels with the aid of cultivation on Drigalski Conradi agar recorded in this connexion that he had found

(*a*) once in a mussel (*Mytilus myosus*), twice in oysters collected from contaminated sea water and also several times in sewer contents a vibrio markedly distinct from *V cholerae* by growing slowly and sparsely in peptone water not liquefying gelatin, and apt to possess 2 or 3 flagella, which was apparently not pathogenic

(*b*) in another species of mussel (*Cardium edule*) a vibrio capable of growing well in peptone water and of liquefying gelatin as well as coagulated blood serum, showing in gelatin stabs growth appearances similar to those of cholera vibrios, but sometimes possessing two flagella and not giving the cholera-red reaction. This organism, called by Klein *Vibrio cardii*, though producing only a local reaction in subcutaneously inoculated guinea-pigs, killed these animals within 20 hours if injected intraperitoneally in doses of one loop of a 24-48 hour agar culture At autopsy the peritoneal cavity was found to contain a viscous and turbid exudate abounding in vibrios the peritoneal coats and the intestines were congested and showed haemorrhages.

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(*a*) once in a mussel (*Mytilus mytosus*) twice in oysters collected from contaminated sea water and also several times in sewer contents a vibrio markedly distinct from *V cholerae* by growing slowly and sparsely in peptone water not liquefying gelatin, and apt to possess 2 or 3 flagella, which was apparently not pathogenic-

(*b*) in another species of mussel (*Cardium edule*) a vibrio capable of growing well in peptone water and of liquefying gelatin as well as coagulated blood serum, showing in gelatin stabs growth appearances similar to those of cholera vibrios, but sometimes possessing two flagella and not giving the cholera-red reaction. This organism, called by Klein *Vibrio cardii*, though producing only a local reaction in subcutaneously inoculated guinea-pigs, killed these animals within 20 hours if injected intraperitoneally in doses of one loop of a 24-48 hour agar culture. At autopsy the peritoneal cavity was found to contain a viscous and turbid exudate abounding in vibrios the peritoneal coats and the intestines were congested and showed haemorrhages.

Similarly Remlinger & Nouri (1908b) stated that they had isolated from oysters and mussels collected at Constantinople a vibrio which because it was agglutinated by a high titre cholera immune serum only in a dilution of 1/100 had to be considered as a cholera like organism. The two workers stressed that such vibrios could be responsible for the appearance of choleriform diarrhoea in man.

In two articles published in 1909 and 1912 respectively Bergman reported (*a*) on the isolation of a cholera like vibrio (*V. anguillarum*) found to be responsible for an infectious tumour disease (*rote Beulenkrankheit*) of eels, and (*b*) on that of a similar or possibly identical organism which was the cause of an infective eye disease (keratomalacia) in codlings (*Gadus morrhua*) and, as shown by one observation, also of a gingivitis terminating in septicaemia in pike

The organisms in question were capable of liquefying gelatin and grew well in peptone water but differed in other respects from *V. cholerae* particularly by not giving a cholera-red reaction and growing best at room temperature

The vibrios isolated from the codlings proved to be experimentally pathogenic not only for this species, for eels carp and roaches, but also for subcutaneously infected crayfish, for 1 subcutaneously and 1 out of 2 intraperitoneally infected mice A few tests with guinea pigs (subcutaneous and intraperitoneal infection) and with intravenously or intraperitoneally inoculated rabbits gave negative results.

Though showing some differences in agglutination tests with sera raised in rabbits with the organisms isolated from codlings, the various strains studied by Bergman belonged obviously to one serological group of fish-pathogenic vibrios.

Defressine & Cazeneuve (1914) demonstrated the presence of cholera like vibrios in 20% of the mussels collected in France at a short distance from the river Neuve which had been found to be contaminated with true cholera vibrios. The cholera like organisms, though identical with *V. cholerae* in regard to their morphology staining, cultural and fermentative properties and found to be pathogenic for guinea pigs and rabbits, differed from classical cholera vibrios by their serological reactions, haemolytic power and inability to reduce nitrates to nitrites.

A further profound study of a cholera-like vibrio responsible for violent and recrudescent epizootics among carp bred in a pond near Vienna, Austria, was made by David (1927) The organisms in question, designated as *V. piscium*, differed from cholera vibrios not only serologically but also in other respects.

Particularly noteworthy in the latter connexion is that they (*a*) failed to give the cholera red reaction (*b*) grew poorly in peptone water and (*c*) showed an aberrant behaviour when cultivated on agar their growth optimum on this medium ranged from 8 C to 20 C Involution forms

plentiful vibrios in the stools, which contained whitish granules, and succumbed after 30 hours. A second lizard had diarrhoea for only 2 days, but continued to harbour the vibrios in its stools until it died in a cachectic condition after a month The other 2 animals showed only passing diarrhoea continuing to excrete the vibrios in their stools for 2 and 5 days respectively

Interesting as these observations are it is difficult to share Goéré s belief that lizards could become carriers of *V cholerae* under natural conditions

Fish and shellfish

The problem of the natural occurrence and persistence of *V cholerae* in aquatic animals is of great practical importance because (*a*) as has been discussed in Chapter 3 this organism is apt to survive for considerable periods or even to multiply in fish and shellfish stored for consumption and (*b*) it has been repeatedly found that such contaminated foodstuffs have been the cause of cholera manifestations in man It deserves attention in this respect that, as recorded by Takano Ohtsubo & Inouye (1926) a report from the sanitary authorities of the Kochi Prefecture in Japan stated

"that cholera vibrios were recovered from a large proportion of the fish caught in the bay which had been polluted with cholera."

More convincing than this information are the observations of Kundu & How (1938), who though as a rule finding only cholera like vibrios in the prawns, lobsters, and shrimps brought in baskets from the delta districts of Burma to the Rangoon market, were in two instances able to isolate *V cholerae* from prawns.

Before further dealing with the fate of cholera vibrios in fish and shell fish it is important to refer to observations demonstrating the presence or even the pathogenicity of cholera like vibrios in these aquatic animals.

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(*b*) in another species of mussel (*Cardium edule*) a vibrio capable of growing well in peptone water and of liquefying gelatin as well as coagulated blood serum, showing in gelatin stabs growth appearances similar to those of cholera vibrios, but sometimes possessing two flagella and not giving the cholera-red reaction. This organism, called by Klein *Vibrio cardii* though producing only a local reaction in subcutaneously inoculated guinea-pigs, killed these animals within 20 hours if injected intraperitoneally in doses of one loop of a 24-48 hour agar culture At autopsy the peritoneal cavity was found to contain a viscous and turbid exudate abounding in vibrios the peritoneal coats and the intestines were congested and showed haemorrhages.

"When fish kept in a glass jar are fed with food containing cholera vibrios the organism can be demonstrated in the intestine of the fish The vibrio is naturally also diffused in the water and becomes attached to the surface of the fish. If such a fish be moved to fresh clean water it is found that the vibrio entirely disappears in 4½ days. Even when the fish is kept in water containing cholera vibrios, the vibrio does not penetrate the skin "

Dealing with the relationship of shellfish to cholera Takano and colleagues maintained that

"When an oyster shelled or unshelled, is placed in fresh sea or salt waters which are contaminated with the cholera vibrio the vibrio enters the stomach within 1 minute When oysters and clams are kept in cholera-polluted sea-water at a temperature of zero to 5°C., the vibrio survives for 1½ months and at 22 C. for 15 to 20 days."

As briefly mentioned by David (1927) in his publication quoted above carp as well as frogs, which he had infected with large doses of cholera or El Tor vibrios cultivated at room temperature succumbed after 4-8 days and showed at autopsy signs identical with those in the animals infected with *V piscium*

Schoebl & Nukada (1935) in order to elucidate the possible role of fish in cholera, infected 8 young carp (body weight 50 g) by administering with the aid of a stomach tube 2.5-ml doses of a peptone water culture of *V cholerae* and killed the fish at intervals ranging from one to 19 days.

Cholera vibrios were invariably found in the 5 carp killed within the first 6 days after infection always in the stomach,[1] usually also in the intestines, once in the bile as well but never in the faeces In one fish killed on the 7th day positive cultures were obtained from the faeces on the same day while in another of the carp sacrificed on the 19th day *V cholerae* had been found in the faeces on the 17th day

The two workers concluded from these observations that fish might serve as cholera carriers However the method of infection used by them was rather unrealistic as well as drastic.

As briefly mentioned by Abraham (1954) suggestive results in experimental cholera infection of *Saurus ophidion*, a small fish commonly known under the name of the Bombay duck, were obtained by Albuquerque & Bhat (1953) whose original publication was not available to the present writer

In an article, to which further reference will be made later on Pillay and co-authors (1954) reported that

"The viability of cholera vibrios in the alimentary tracts of certain fishes was studied by the technique of artificial infection. Specimens of the Climbing Perch (*Anabas testudineus*) and Murrel (*Ophicephalus punctatus*) which were kept under observation and had been found to have ceased to excrete N A G vibrios [i.e. cholera-like vibrios], were infected by feeding them on artificially infected pupae and larvae of house-flies bred in the laboratory Each day the fish were removed to fresh sterilized aquaria and the water samples from the old aquaria were examined. But no vibrios were recovered for a period

[1] Schoebl & Nukada maintained in this connexion that the stomach contents showed in most fish species strongly alkaline reaction, whereas the intestinal contents were neutral in reaction.

alone developed on the cultures kept at 37 C and no further growth took place when subcultures made from such slants were kept at the same temperature in the incubator If on the contrary the subcultures were held at room temperature morphologically normal and subcultivable vibrios developed

The *V piscium* was not pathogenic for guinea pigs, and as a rule also not for rabbits, and produced no fatal infection in intramuscularly inoculated pigeons, but was experimentally pathogenic for carp also for a few other fish species and, as mentioned above for frogs

Reference has already been made in Chapter 2 to the isolation from fish and shrimps caught at Rostov-on the-Don as well as from the water of that river of cholera-like vibrios, which were found to be responsible for the causation of a choleraic disease in man.

To avoid duplications, further laboratory observations on the occurrence of cholera like vibrios in fish and shellfish recently made by workers in India will be recorded when dealing in the tenth chapter with the role of these animals in the epidemiology of cholera.

Experimental observations on the fate of cholera vibrios in fish and shellfish seem to have been made first by Remlinger & Nouri (1908a)

These workers put goldfish into large jars filled with water to which cholera vibrios were added 48 hours afterwards the fish were killed and cultures were made from their intestinal tract and their meat. It was invariably possible to isolate the vibrios from these materials in quantities corresponding to the degree of contamination of the water Cultures from infected fish which had been cooked, fried, or grilled, proved, on the other hand, always sterile

Kabeshima (1918b) noting that (*a*) cholera in Japan broke out mainly on the coast where, by preference, it attacked fishers and (*b*) Okawa had demonstrated the presence of *V cholerae* in fish (*Trichluris leptmus*) loaded on a fishing vessel in the course of an epidemic, felt impelled to explore the possible role of aquatic animals in cholera with the aid of experiments.

Kabeshima put for this purpose sea fish and shellfish of several species into a tank containing sea water to which 50 mg of cholera vibrios harvested from 18-hours-old agar cultures were added. After the animals had been kept in the tank for varying periods, they were kept immersed for 10-15 minutes in a 2 per 1000 solution of mercuric bichloride and then left wrapped in a cloth soaked in the same fluid until they died. Autopsy was made after the fish had been cleaned with ethanol and the viscera were put into large tubes with peptone water which were incubated for about 15 hours. Platings were then made from the topmost layers of the fluid cultures.

Experimenting in this manner with 24 scombridae, 8 shrimps, and 20 others species, Kabeshima found that a penetration of *V cholerae* into the intestine had taken place in 76.9 / of the animals after a sojourn in the contaminated water for periods ranging from as little as 5-10 minutes up to 5 hours. He likewise found cholera vibrios in the intestines of 17 out of 37 fish or shrimps left dead for 1 5 hours in the contaminated water and postulated that in these instances the anus had been the portal of entry of the infection.

In their studies on cholera in Japan Takano Ohtsubo & Inouye (1926) besides referring to the work of Kabeshima, stated

Tizzoni & Cattani (1886 1888) collected in 1885 three lots of 15-20 flies each in a cholera hospital decapitated these insects 3-4 hours later and used their bodies for cultivation in fluid blood serum In two out of these three tests they could subcultivate vibrios which showed, morphologically and culturally properties identical with those of *V cholerae*

As Simmonds (1892a) stated in a short note he had (*a*) succeeded in growing numerous *V cholerae* colonies in gelatin cultures inoculated with flies, which 5-45 minutes previously had been brought in contact with the intestine of a cholera victim and (*b*) obtained identical results with 6 flies which had been kept for a period of $1\frac{1}{2}$ hours between the time of such an exposure on a cholera intestine and cultivation

In order to furnish clear proof that flies which came in contact with cholera-contaminated materials actually fed on them Sawtschenko (1892) disinfected such insects after their exposure on cholera cultures or on cholera faeces, or on the intestinal contents of cholera victims by (*a*) rinsing them with an antiseptic fluid (*b*) washing them in alcohol and (*c*) drying them first with the aid of filter paper and then by quick passage through the flame of a Bunsen burner The posterior end of the fly s body was then clipped off with sterile scissors and material for cultivation on gelatin plates was taken from the abdominal cavity with a platinum loop Sawtschenko obtained in this manner positive results with the abdominal contents of flies tested 1-4 days after infection. As shown by guinea pig experiments, the virulence of the cholera vibrios had not been abated through a sojourn in the intestines of the flies for 2 or 3 days. Some evidence was obtained to show that the organisms multiplied in the intestinal tract of the insects.

Uffelmann (1892) brought cholera infected flies in contact with either freshly boiled milk or a small piece of fried beef Cultures made from these materials (1) immediately after contamination by the flies, and (2) after the milk and beef had been kept for 16 and 24 hours respectively invariably gave positive results However the number of cholera colonies developing on the plates which had been inoculated with the stored materials was most markedly larger than that on the immediately inoculated plates

It will be noted that the interesting experiments of Uffelmann confirmed the validity of Grassi s pioneer observations.

Tests resembling those of Sawtschenko were made with houseflies by Craig (1894) apparently the first worker in this field in the USA He fed 3 large flies for 3 days on a piece of bread which had been moistened with a cholera broth culture After the insects had been killed, their wings and legs were cut off The hinder part of their bodies was then squeezed with a sterile forceps and the small drop of faeces appearing in the anus was used for the inoculation of broth cultures The growths thus obtained were contaminated and gave a negative cholera red reaction However in one of the three instances a pure strain of *V cholerae* could be subcultivated,

of more than 30 days. The fish were then dissected and their intestinal content examined bacteriologically Haemolytic non-agglutinable vibrios were recovered."

They added that in 4 sets of subsequent experiments climbing perches, artificially infected with cholera vibrios, were found to excrete cholera like vibrios of Heiberg s Group II for a period ranging from 2 to 4 days after infection They claimed that these observations "suggest the possibility of mutational changes of *V cholerae* in the alimentary tracts of at least certain fishes"

An excellently documented study on the viability of cholera vibrios in clams (*Meretrix casta*) was recently published by Abraham (1954) who resorted (*a*) to exposure of such clams for half an hour in water to which saline suspensions of the centrifugate of 8-hour-old cholera cultures had been added, and (*b*) to inoculation tests made by injecting 0 05-0 1 ml of suspensions of cholera vibrios containing 14 000 million of the organisms per millilitre into the foot of the animals Daily examinations of specimens of the infected clams which after adequate cleansing were kept in running water showed that cholera vibrios were apt to survive in these animals for 3 days without multiplication and that the strains recovered from them did not show any evidence of mutation

The epidemiological significance of the above recorded and allied observations will be discussed in Chapter 10

OBSERVATIONS ON INSECTS

Common flies

Though as discussed by Sticker (1912 [1]) the existence of a causal connexion between an abundance of flies and the spread of cholera epidemics had been postulated long before Koch s time, naturally definite proof for this assumption could be obtained only after the discovery of *V cholerae* Grassi (1884) apparently the first worker to adduce such proof did so in an indirect manner by demonstrating the presence of the "curved" bacilli in substances such as sugar and fruits on which flies had alighted after they had been in contact with cholera stools.

The validity of the contention made on account of these observations by Grassi, that flies were of importance in the spread of cholera, was confirmed in 1885 by Maddox through the demonstration of the regular presence of *V cholerae* in the faeces of flies which had been permitted to feed on sugar solutions contaminated with cholera cultures The findings made by Maddox were soon confirmed in their turn by several other workers (see summaries by Sticker 1912 and Schuckmann 1926) Significant results obtained by these early observers may be summarized thus.

[1] See paragraph 79 of his work.

the organisms on their surface but only in rare instances in their intestine or faeces (3) pupae did not carry cholera vibrios, and (4) adult flies reared from larvae which had been kept in cholera faeces were found to be free from *V cholerae*

The results of more recent studies on the problem presently under review by workers in India may be summarized as follows.

A limited number of excellently planned experiments with laboratory bred flies originally derived from latrines and infected by feeding them on cholera milk-emulsions was made by Gill & Lal (1931) Examinations by cultural methods (enrichment in peptone water followed by plating on Esch s medium and subcultivations for identification tests) were made of the following materials

(1) pieces of sterilized meat on which the infected flies had rested for a few minutes

(2) sterilized milk filled into capillary tubes and contaminated by the infected flies through insertion of their proboscis for the purpose of feeding

(3) faeces of the infected flies deposited in sterilized tubes

(4) whole chloroformed and crushed flies

(5) crop and gut of the infected flies, excised under sterile precautions

Summarizing their findings, the two workers stated that

"The experiments recorded above are admittedly meagre and it is not at present claimed that they justify the conclusion that a true host-parasite relationship exists between the fly and the vibrio It would, however seem in the first place that the vibrios are capable of surviving in the fly for a period of at least five days. Secondly it would appear that the cholera vibrio apparently disappears from the body of the fly after 24 hours or so but that it re-appears on or about the fifth day at which time the fly is capable of infecting food [i.e. meat] by its faeces. Thirdly it has been shown that infection of milk via the proboscis can take place up to 24 hours, but it has not yet been proved that infection via the proboscis can take place on and after the fifth day"

Gill & Lal suggested on account of these observations

that possibly one phase of the life-cycle of the cholera vibrio is passed in the body of the house-fly and that this insect may play a more important part in the transmission of cholera than has hitherto been suspected"

Results of experimental studies made in the King Institute Madras (see Soparkar 1938) indicated that cholera vibrios remained viable in flies for not longer than 4 hours at most—a length of survival found in only 2 out of 60 observations. However a survival of the organisms in deposited faeces and vomits of the infected flies for 8 hours was frequently noted and occasionally the vibrios remained cultivable from these materials for as long as 24 hours.

As will be gathered from the above recorded findings made since 1884 the epidemiological significance of which will be appreciated in a subsequent

identified by typical growth appearances in gelatin stab cultures, and a positive cholera red reaction.

Observing a cholera outbreak involving part of a jail in India, Macrae (1894) was able to isolate *V cholerae* from samples of milk exposed to the flies which abundantly infested the affected premises. These were separated from the other half of the jail by a high wall beyond which the flies evidently could not pass It was probably for this reason that the infection remained restricted to the originally invaded section of male prisoners.

Working during a cholera outbreak in North China, Tsuzuki (1904) was able (*a*) to cultivate cholera vibrios from flies collected in a house the inmates of which had been affected by cholera and (*b*) to demonstrate that flies were capable of carrying within a period of 24 hours the organisms from an agar culture of *V cholerae* to a sterile dish exposed near to this culture

As summarized by Sticker (1912) Ganon (1908) working in the Nether lands East Indies,

"demonstrated in numerous experiments that flies ingest the cholera vibrio from faeces and cultures, transfer it in a viable and multipliable form to water pisang and rice, and [then] die themselves after 2-4 days" [Trans.]

Experimenting with old stock cultures, Graham-Smith (1910) found that the cholera vibrios (*a*) rapidly succumbed on the wings and legs [1] of flies and (*b*) survived in the crop and intestinal tract of these insects not longer than 48 hours, being demonstrable in their faeces up to 30 hours after infection.

In the course of a further study Graham-Smith (1911) established that blowflies developing from larvae which had been fed on meat contaminated with *V cholerae* were free from infection.

Passek (1911) a Russian worker experimenting with houseflies and bluebottle flies, found cholera vibrios to be most abundant in the intestinal contents of these insects 12 24 hours after infection and to be absent after 72 hours. In the intestines of adult flies *V cholerae* was usually present in association with *Proteus vulgaris* a symbiosis which, in the experience of Passek, greatly enhanced the virulence of the cholera vibrios. A corollary to this was that the vibrios isolated from the intestines of flies just hatched, in which the symbiont was still absent, had a lessened virulence Highly virulent vibrios associated with *Proteus* could be isolated from the faeces of adult flies up to 3 hours after voiding.

Interesting observations by Alessandrini & Sampietro (1912) showed that (1) flies which had been brought in contact with cholera-contaminated materials abundantly harboured the organisms on their surface as well as in their intestines for 24-36 hours (2) contaminated fly larvae also showed

[1] It should be noted in this connexion that in the experience of Chantemesse & Borrel (1905) *V cholerae* remained viable on the legs of flies up to 17 hours after contact with cholera materials.

24 to 48 hours. Though apparently not multiplying in the cockroaches, the cholera vibrios passing through these insects underwent no loss in virulence. The faeces of the infected cockroaches, if kept in a moist environment, e.g. when placed on fresh beef, lettuce fish or clams at room temperature contained viable cholera vibrios for at least 16 hours. Numerous cholera vibrios could be found in the vomits of infected cockroaches voided up to one hour after infection

Observations similar to those of Barber were made by Toda (1923) who experimented with another cockroach species, *Blatta germanica*. He found that 14 out of 94 of these insects, when fed with bread soaked in a suspension of *V. cholerae* harboured viable vibrios in their faeces for periods up to 72 hours. In one of these cockroaches cholera vibrios could still be isolated from the intestine when the insect in question was killed 120 hours after infection

Other insects

As briefly mentioned by Nuttall (1899) Maddox (1885) experimented not only with flies (see above), but also with bees, a wasp and a beetle and found in the faeces of these insects also after they had been fed with cholera-contaminated sugar solutions, motile organisms morphologically identical with *V. cholerae*

Further observations made regarding the fate of cholera vibrios in insects other than flies or cockroaches may be summarized as follows

(1) *Ants*. Experimenting with cholera-infected red ants (*Monomorium latinode*), Barber (1914) was unable to obtain satisfactory results with the insects' faeces, which became rapidly dry. He noted, however that the ants readily fed on cholera stools as well as on cholera cultures and that the ingested organisms could be recovered from the crushed bodies of such insects killed 8-9 hours after infection.

(2) *Beetles*. As noted above, Cao (1898) found that cholera vibrios were apt to pass through the intestinal tract of certain beetles (e.g. *Tentyria sardoa, Blaps mucronata Pimelia bifurcata*, and *Pimelia sardoa*) in a viable and virulent condition.

(3) *Caterpillars*. Metalinkow & Gaschen (1921) stated in a short communication that the caterpillars of the bee moth *Galleria* were susceptible to parenteral infection with *V. cholerae* but could be easily and rapidly immunized against this either by the injection of sublethal doses of living vibrios or by vaccination with suspensions of the organisms which had been killed by heating at 58 C.

PATHOLOGY OF HUMAN CHOLERA

Morbid Anatomy

As can be gathered from a survey of the early literature and a study of compilations such as that of Sticker (1912) the morbid anatomy of cholera had been the subject of numerous and often extensive investigations long before the discovery of *V. cholerae* particularly after the disease began to make inroads into Europe, where ample facilities for such work existed

chapter the flies are apt to serve as vehicles and even as short-term repositories for *V cholerae*, but do not serve as reservoirs of the infection for prolonged periods, as has been suggested by some writers. There is also no valid reason to assume that the cholera vibrios undergo mutations in the flies. On the contrary, a special study made in this respect by Lal and colleagues (1939) failed to show changes in the biochemical reactions and in the serological properties of the strains recovered from experimentally infected flies (genus *Musca*)

Phoridae

The possibility of a spread of cholera by *Aphiochaeta ferruginea*, a dipterous insect belonging to the family of Phoridae, was experimentally explored in the Philippine Islands by Roberg (1915) He stressed in this connexion that this fly which had a wide distribution throughout the tropical regions and some adjacent parts of the temperate zone but was apt to be overlooked on account of its minute size, was potentially dangerous because (*a*) it bred in putrefying materials, preferably in human faeces, and (*b*) it was capable as a result of its omnivorous feeding habits of contaminating food for human consumption or even of becoming ingested together with such food.

Roberg found that the larvae of this fly

"when fed on a medium containing cholera vibrios harbor these organisms in their intestinal tracts only as long as cholera vibrios are present in the medium and sufficiently plentiful in numbers not to be outgrown by other organisms.

"There is a transference of vibrios from the larvae to the pupae and from the pupae to the imagines. This, however is only possible when the larvae and the pupae are constantly changed to a sterile medium and drenched with 24-hour-old broth cultures of cholera vibrios. If this is not done, the vibrios are outgrown by the bacteria which are commensals in the intestines of the larvae and by the bacteria which are associated with putrefaction of the medium."

While under these circumstances the larvae of this fly species seem incapable of playing a role in the transmission of cholera, it deserves attention that in the experience of Roberg adult *Aphiochaeta ferruginea* when fed on cholera-contaminated materials, harboured the organisms on their surface for 10 hours and in their intestinal tract for 26 hours.

Cockroaches

Early experiences of Cao (1898) to the effect that cholera vibrios, when passed through the intestinal tract of cockroaches (*Periplaneta orientalis*) as well as through other insects (flies or beetles) remained viable and virulent, have been elaborated through further observations of Barber and Toda.

Barber (1914) experimenting with winged adults of *Periplaneta americana* kept at temperatures of 29 31 C, established that if these insects were fed on human cholera stools, the organisms could be isolated from their faeces from 6 to at most 79 hours after infection usually from

no evidence could be found of any abatement of the virulence of *V cholerae* in the course of the outbreaks.

While for the reasons stated above one must insist that the gross post mortem findings met with in a sufficiently large random sample of cholera victims show a marked variability, it must be stated at the same time that the features met with at autopsy of sufferers who succumbed in the algid stage of the disease though not pathognomonic, are rather characteristic. Crowell (1914) aptly stated in this connexion that the signs manifest in such victims were those of an

"acute catarrhal enteritis associated with (1) cyanotic finger nails (2) dry tissues (3) oligemia (4) dry and sticky peritoneum with pink serosa of ileum (5) contracted and empty urinary bladder (6) shrunken dry spleen and liver (7) acute degeneration of parenchymatous organs (8) poorly coagulated blood (9) absence of formed faeces (10) presence of rice-water intestinal contents (11) prominence of lymphoid tissue in ileum"

These and other signs met with at autopsy of cholera victims may be described in some detail as follows.

General appearances

As excellently described by Liebermeister (1896) in the case of individuals who succumbed to cholera quite early or died during the algid stage

"the whole body appears to be shrunken, the skin is withered and wrinkled, the eyes are sunk in, are usually half open and show signs of desiccation on the uncovered portions, the nose is pointedly prominent, the cheeks are sunk in, the malar bones markedly prominent. The fingers are curved, the arms and legs in a flexed position with markedly prominent muscle bellies (so-called fencing posture) The cyanosis present during life is generally manifested by a dark, more greyish colouration of the body surface, [but] many portions of the skin still show an injection of the finer veins and are therefore bluish-grey to violet, the lips, nails, fingers, and toes in particular still showing definite cyanosis. Rigor mortis appears early becomes marked and lasts long during its development one may observe often clearly perceptible movements of the limbs, particularly the fingers. A postmortal increase of the body temperature may be observable the dead bodies cool slowly Presumably on account of the decreased water-content of the body putrefaction sets in late." [Trans.]

These marked signs are less conspicuous or even altogether absent in the case of victims succumbing during later stages of cholera when, owing to the disappearance of the dehydration and grave circulatory disturbances the general aspect of the dead bodies shows nothing characteristic. While rigor mortis does not become marked in such cases, putrefaction is apt to set in rapidly and to reach an intense degree

It is generally held that the exanthemata sometimes present in cholera sufferers during life are no longer visible after death. On the contrary the presence of skin haemorrhages has been noted at the autopsy of such victims by several modern observers, Simmonds (1892b) referring even to one instance in which signs of purpura were manifest. It is noteworthy however that Stoerk (1916) who dissected 373 cholera victims, referred to the

Being for a period of more than five decades unable to correlate their autopsy findings with, and to confirm them by bacteriological examinations the early workers naturally aimed at discovering macroscopic features which were pathognomonic. The existence of such fully characteristic post mortem signs was often postulated Indeed it is curious to note that claims to this effect were made as late as in 1892 by Klebs in a review of the ample experiences gained in his past studies on the morbid anatomy of cholera In his opinion, a "very certain diagnosis" of early cholera manifestations could be made owing to the presence of (1) a greyish white and firmly adherent layer of mucus covering the mucosa of the small intestine, and (2) immediately appearing alterations in the kidney Klebs insisted therefore that anti-cholera measures should be instituted as soon as such autopsy features had been met with without waiting for the results of bacteriological examination, which might come to hand late or might even be negative.

The validity of these contentions was emphatically denied by Simmonds (1892b), who asserted that it was impossible to arrive at a final post mortem diagnosis of cholera without bacteriological examinations and that it was consequently not permissible to use the macroscopic findings alone for an identification of initial cases.

The opinion that as Crowell (1914) aptly expressed it, the bacteriologist was "the court of last appeal" in the diagnosis of cholera, has been upheld by almost all modern pathologists

A further most important fact established through the correlation of laboratory investigations with the macroscopic findings made at the autopsy of cholera victims was that, in contrast to the impression given by most early descriptions, the gross changes produced by *V cholerae* in man were characterized by a marked variability rather than by uniformity

Crowell (1914) devoting attention to this fact in his short, but excellent, study on the diagnosis of Asiatic cholera at autopsy stated with much reason that this variance of the post mortem signs was due to

"(1) the stage of the disease at which the patient died (2) whether or not the patient had received treatment, and (3) the presence of associated diseases"

It was also noted by some observers, e g. by Simmonds, that early in cholera outbreaks most of the victims showed signs suggestive of their death in the first stages of the disease particularly during the algid stage whereas later in the epidemics an increasing number of the dissected individuals showed evidence of having survived longer It is probable that changes of this nature were due mainly to gradual improvements of the anti-epidemic work, owing to which an increasing number of the sufferers received treatment with initially beneficial results, many however succumbing afterwards to uraemia or other complications This at least was the experience of the present writer in the cholera outbreaks in China, where

no evidence could be found of any abatement of the virulence of *V cholerae* in the course of the outbreaks.

While for the reasons stated above one must insist that the gross post mortem findings met with in a sufficiently large random sample of cholera victims show a marked variability, it must be stated at the same time that the features met with at autopsy of sufferers who succumbed in the algid stage of the disease though not pathognomonic, are rather characteristic. Crowell (1914) aptly stated in this connexion that the signs manifest in such victims were those of an

"acute catarrhal enteritis associated with (1) cyanotic finger nails (2) dry tissues (3) oligemia (4) dry and sticky peritoneum with pink serosa of ileum (5) contracted and empty urinary bladder (6) shrunken dry spleen and liver (7) acute degeneration of parenchymatous organs (8) poorly coagulated blood (9) absence of formed faeces (10) presence of rice-water intestinal contents (11) prominence of lymphoid tissue in ileum"

These and other signs met with at autopsy of cholera victims may be described in some detail as follows.

General appearances

As excellently described by Liebermeister (1896) in the case of individuals who succumbed to cholera quite early or died during the algid stage

"the whole body appears to be shrunken, the skin is withered and wrinkled, the eyes are sunk in, are usually half open and show signs of desiccation on the uncovered portions, the nose is pointedly prominent, the cheeks are sunk in, the malar bones markedly prominent. The fingers are curved, the arms and legs in a flexed position with markedly prominent muscle bellies (so-called fencing posture) The cyanosis present during life is generally manifested by a dark, more greyish colouration of the body surface, [but] many portions of the skin still show an injection of the finer veins and are therefore bluish-grey to violet, the lips, nails fingers, and toes in particular still showing definite cyanosis. Rigor mortis appears early becomes marked and lasts long during its development one may observe often clearly perceptible movements of the limbs, particularly the fingers. A postmortal increase of the body temperature may be observable the dead bodies cool slowly Presumably on account of the decreased water-content of the body putrefaction sets in late." [Trans.]

These marked signs are less conspicuous or even altogether absent in the case of victims succumbing during later stages of cholera when owing to the disappearance of the dehydration and grave circulatory disturbances the general aspect of the dead bodies shows nothing characteristic. While rigor mortis does not become marked in such cases, putrefaction is apt to set in rapidly and to reach an intense degree

It is generally held that the exanthemata sometimes present in cholera sufferers during life are no longer visible after death. On the contrary the presence of skin haemorrhages has been noted at the autopsy of such victims by several modern observers, Simmonds (1892b) referring even to one instance in which signs of purpura were manifest. It is noteworthy however that Stoerk (1916) who dissected 373 cholera victims, referred to the

total absence of skin haemorrhages even in cases where copious haemorrhages were present on the serous membranes.

Ciaccia (1914) laid great stress upon the presence in the dead bodies of cholera victims of a "skin sign" consisting of

"a peculiar stiffness of the skin, on account of which the latter appears to adhere to the aponeurotic layers and cannot be lifted easily in folds" [Trans.]

He claimed that this sign, while absent in the dead bodies of individuals succumbing to other diseases was invariably present in cholera victims and, therefore, pathognomonic. Since, however Ciaccia felt entitled to include in his series of cholera cases some which gave bacteriologically negative results, it is not possible to share his belief in the infallibility of the skin sign

Subcutaneous tissues and musculature

In individuals who succumbed to cholera while dehydration and general circulatory disturbances persisted, the subcutaneous tissues are found to be firm and dry the muscles dark red.

Boltz (1893) was apparently the first modern worker to draw attention to the presence of different degrees of degeneration in the musculature of the larynx, the diaphragm, and occasionally the calf muscles of cholera victims. While varying in degree in the different muscle groups in individual cases, generally speaking, the degeneration of the muscles appeared to be most frequent and marked in the victims succumbing to the disease with moderate rapidity less conspicuous in those who succumbed quite rapidly or later in the disease Boltz maintained that these degenerative processes were the result of a direct action of the cholera toxin on the protoplasm of the muscles.

Further referring to the condition of the musculature in cholera victims, Crowell (1914) stressed the importance of the rigidity of the abdominal muscles, which showed evidence of the waxy degeneration previously described in the case of typhoid fever by Zenker (1863)

Stoerk (1916) while confirming the presence of degenerative processes in the larynx muscles and also in other parts of the musculature in the dead bodies of cholera victims through exhaustive investigations, was unable to establish a relation between the degree of these alterations and the duration of the disease. Marked changes were sometimes met with after a "strikingly short" duration of illness, while those met with in some cases of longer duration were quite inconspicuous. Warasi (1922) also noted a rapid appearance of cloudy swelling and waxy degeneration in the abdominal and calf muscles of cholera victims and felt convinced that these alterations were the result of an action of the cholera toxin. The presence of various atrophic changes in the striated muscles of cholera victims was noted by Utsumi (1922), who also drew attention to a waxy degeneration of the heart musculature

Central nervous system

As maintained by most modern observers the alterations met with in the central nervous system of cholera victims are not marked According to the statements usually made in this connexion the sinus of the dura mater and the meningeal vessels were filled with dark thick blood and in individuals succumbing at an early stage the meninges showed dryness or were at most slightly oedematous while little fluid was present in the ventricles of the brain The occasional presence of meningeal haemorrhages was also recorded It was further noted that in individuals succumbing during later stages of cholera meningeal oedema was apt to be conspicuous and that in such cases considerable accumulations of fluid could be present in the brain ventricles

In marked contrast to these descriptions Michailow (1909 1912, 1913) (*a*) pointed to observations made by some earlier workers of more conspicuous alterations in the nervous system of cholera victims and (*b*) recorded that he himself had made identical findings, including far gone degeneration of nerve cells and nerve fibres in the brain medulla oblongata, and spinal cord swelling of the endothelial cells of the blood vessels of these parts, and hyaline degeneration of the vessel walls as well as proliferation of the ependyma of the central canal of the spinal cord He stated moreover that he had twice demonstrated the presence of organisms morphologically identical with *V cholerae* near degenerated nerve cells of the brain or the spinal cord However Michailow admitted in his elaborate 1913 study that he had been unable to follow up the latter observations which had been made with the aid of histological examinations only and that consequently the problem of these organisms had remained " unelucidated and unsolved " There can be no doubt, however that otherwise the results of Michailow s painstaking studies deserve attention

Reference to the presence of meningitis in cholera victims has been made by a few observers but there can be hardly any doubt that as a rule the occurrence of such inflammatory processes has been the result of intercurrent infections or of post-choleraic invasions of organisms other than the *V cholerae* It would appear however that the case of an acute meningitis in a cholera affected child referred to by Scicluna (1912) in a report the original of which was not accessible to the present writer formed an exception to this rule.

Mouth and fauces

The alterations found in the mouth and fauces of cholera victims succumbing early in the disease usually consisted of marked congestion associated with dryness of the mucous membranes Moreover as noted by Rogers (1921) enlargement of the lymph follicles at the back of the tongue and in the pharynx was apt to be present No significant alterations have

been recorded in the buccal and pharyngeal cavities of sufferers dying in the later stages of cholera

Thyroid gland and thymus

The thyroid gland and thymus of cholera victims were often found to be congested. In addition to this feature, Banerjee (1939) was struck by the frequency with which the latter organ was enlarged, but the significance of this condition, often noted by the present writer in pneumonic plague victims as well, is not clear

Respiratory system

While the mucosa of the air passages from the larynx to the bronchioles is usually markedly reddened in the victims who succumb late to cholera this feature is less conspicuous, and according to Sticker (1912) even absent, in the case of individuals dying in the early stages of the disease. While then but little viscous mucus is found in the air passages, as a result of catarrhal conditions developing in the course of the disease, copious mucoid or even purulent matter may be present in the trachea and bronchi of persons who fell a prey to cholera after more prolonged illness. More marked alterations, eg. laryngeal ulcers or glottis oedema, have been recorded occasionally e g. by Simmonds (1892b)

Shortly but adequately describing the condition of the lungs in cholera victims Crowell (1914) stated

"The lungs are as a rule poorly inflated, and the pleura is exceedingly dry In cases which have lost much fluid, the cut surfaces of the lungs are red but very dry while in the very early cases and in those which have received fluid, congestion and oedema of the lungs may be marked."

Like other observers Crowell further found that quite frequently ecchymoses of the pleurae may be present in cholera victims

In the experience of most workers who had opportunities of dissecting considerable numbers of cholera victims, pneumonic processes formed a frequent complication in the later stages of the disease. Thus Simmonds (1892b) recorded that he had found pneumonic foci, almost always of a lobular character 62 times in 150 autopsies of cholera victims who had succumbed after an illness of more than 3 days Similarly Stoerk (1916) besides noting the presence of small haemorrhages in the lung parenchyma of dead bodies also showing pleural and epicardial ecchymoses, maintained that pneumonias, mostly of a lobular character formed the most frequent complication of cholera.

There can be no doubt that as a rule not *V cholerae* but other secondarily invading bacteria were responsible for these lung complications. However in marked contrast to this usual evolution, Greig (1912, 1913c, 1914a) stated (*a*) that he had demonstrated the presence of *V cholerae* in pneumonic foci found in 5 cholera victims, one of whom had succumbed

after an illness of only 29 hours, while the others had died after 3 12 days and (*b*) that he had also cultivated cholera vibrios from the lungs of 6 cholera victims who while not manifesting pneumonic foci showed evidence of a generalization of the infection Further reference to these unusual findings will be made in a later part of the present disquisition

Circulatory system

One of the most marked and at the same time most important alterations manifest in cholera victims succumbing during the algid stage as well as during the life of such sufferers is a profound disturbance of the blood distribution as a result of which the blood is accumulated in the large venous trunks and the vessels of the viscera whereas the vessels of the teguments are well nigh empty Chatterjee (1939b) aptly stated in this connexion that cholera shock, instead of solely being the result of dehydration, was also due to the marked dilatation of the capillaries of the internal organs, the victims as it were bleeding into these vessels.

While most older writers describing the circulatory disturbances early manifest in cholera victims often referred to a macroscopically noticeable thickening of the blood, the aspect of which they sometimes likened to that of blueberry jam (see Stucker 1912) modern observers failed to confirm these findings Thus Stoerk (1916) maintained that

"In the heart cavities as well as in the large veins I failed without exception to perceive the inspissation [*Eindickung*] of the blood noted by a majority of the writers at least this phenomenon did not seem recognizable with the aid of the usual anatomical method of judgement" [*Trans.*]

Simmonds (1892b) stated in this connexion more succinctly that in cholera there existed no macroscopically recognizable thickening of the blood but merely an unequal distribution

In cholera victims dissected after death in later stages of the disease the abnormal distribution of the blood described above is less conspicuous or even altogether absent.

If the sufferers died in a dehydrated condition, the pericardial serosa as well as the pericardial cavity exhibited signs of dryness Subserous haemorrhages on the inner layer of the pericardium though occasionally noted are met with but rarely Epicardial haemorrhages on the contrary have been frequently observed Simmonds (1892b) who paid special attention to their occurrence, noted that the epicardial haemorrhages were most often present on the posterior aspect of the heart, particularly near the base In the case of victims who succumbed in the algid stage, these haemorrhages were punctiform sharply defined, and bright red, while in individuals who had died after a more prolonged illness, they were more diffuse and bluish in colour and were finally apt to be quite pale. The occurrence of subendocardial ecchymoses appears to be exceptional.

As can be gathered from the descriptions of most pathologists, the left ventricle and atrium of the heart of cholera victims were contracted, while the corresponding parts on the right side of the organ were filled with dark red blood and/or cruor Simmonds, while considering these features not constant, found the right parts of the heart well filled with blood in the victims who had been treated with intravenous infusions

In the victims who succumbed after a more prolonged illness, more or less pronounced signs of degeneration of the heart muscles—cloudy swelling or as occasionally noted by Fraenkel (1893) even fatty degeneration—were met with invariably The occurrence of endocarditis (caused no doubt by secondary infections) though occasionally noted, for instance by Stoerk (1916) appears to be quite exceptional. As a rule, the heart of cholera victims merely shows the signs usually met with in other acutely infectious diseases as well.

Oesophagus

As described by Sticker the oesophagus of cholera victims

" is usually markedly pale with a lustreless white or reddish tint the longitudinal folds in the lumen are obliterated, the epithelium is often markedly swollen In later stages the mucosa is apt to show a darker hue and then exhibits a marked development of the mucous follicles " [Trans.]

Stoerk drew attention to the presence of small erosions of the mucosa in the lowest portion of the oesophagus due perhaps to the stresses during repeated vomiting. He also recorded an exceptional instance of mucosa necrosis in the lower two thirds of the oesophagus which, however was apparently due to a secondary pneumococcal infection

Stomach

The alterations met with at autopsy in the stomach of cholera victims are apt to show much variation and may be quite inconspicuous. Almost always, however even in the case of sufferers who have succumbed during the first stage of the disease, the stomach, instead of being empty and contracted, has been found to contain at least a small amount of viscous fluid masses Many early observers, probably because they had far more occasion than the modern workers of dissecting cholera victims who had died soon without being treated, commented upon the frequency with which in addition to the above-described fluid contents food remnants were found in the stomach. The reaction of the stomach contents was found to be either alkaline or particularly in the case of deaths having taken place early still acid (Fraenkel, 1893)

The stomach mucosa was usually found to be covered with viscous mucus and somewhat swollen. Congestion of the mucosa was apt to be

marked in the case of victims having succumbed late Smaller or larger submucous haemorrhages have been found to be common Exceptionally necrotic or gangrenous mucosa lesions have been met with more recently by Deycke (1893) and by Crowell (1914)

Small Intestines

Inspecting small intestines of cholera victims who have succumbed in the early stages of the disease one may sometimes note the presence of intus susceptions of one or several intestinal loops which, similarly to those not rarely observed in the dead bodies of children succumbing to various diseases, obviously formed during the agonal period

Palpation of the unopened intestinal loops often gives the impression of an increased thickness and heaviness of the walls which besides being caused by congestion is also largely due to the presence of marked oedema

Like the serous membranes of dehydrated cholera victims in general the serosae of the small intestines in particular are often covered by a sticky and slimy coat, owing to the presence of which the intestinal loops may be glued together It has to be noted however, that this stickiness of the intestinal serosae typical and frequent though it is, is not invariably conspicuous at autopsy of cholera victims succumbing early in the disease Stoerk (1916) insisted in this connexion that the stickiness of the serous membranes, particularly that of the peritoneal coats was best demonstrable when dissections had been made within a few hours after death He often noted then the presence of a quite thin whitish layer covering the peritoneal serosae but felt uncertain whether this macroscopic feature stood in con nexion with the proliferation of the serosa epithelium demonstrable histo-logically in a majority of freshly dissected victims to cholera, but also found in individuals who had succumbed to other acute or subacute intestinal affections

Several other observers, e g. Crowell (1914) and more recently Banerjee (1939) and Chatterjee (1939a) pointed to the practically far more important fact that the stickiness of the intestinal serosae as well as of other serous membranes was apt to be absent in the bodies of cholera victims whose dehydrated condition had been counteracted through treatment with saline infusions.

A second important alteration often met with when inspecting the small intestines in acutely fatal cholera is a rather peculiar pink coloration of the intestinal serosa, due to an intense injection of the blood capillaries, which may involve either the whole small intestine or only its lower parts As maintained by Stoerk this coloration which is all the more conspi cuous because the serosae of the stomach and large intestine are as a rule pale, is well marked only in the freshly dissected dead bodies whereas in the later dissected the pink colour may have faded or may be even merely indicated by a faint congestion of the subserous small veins

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" As a rule it is a grey not unpleasantly smelling or almost odourless fluid, in which membranes very frequently float which are whitish in colour (or sometimes yellowish owing to absorption of bile) delicate and irregularly contoured and which are very small or small but may sometimes be rather larger (up to almost an inch in diameter) They are sometimes found more or less singly and sometimes in great number and they still show by their flat appearance their original relation to the mucosa surface Microscopically they consist mainly of cellular elements glued together by delicate or more abundant masses of mucus. These cellular parts may be aggregates of detached, more or less unchanged, or at times necrotic, intestinal epithelium—almost exclusively from the epithelial coating of the villi of the small intestine—or one may be confronted by complexes of subepithelial round cells lymphoid round cells, polymorphonuclear forms, or occasionally pus cells. In some cases, moreover one finds erythrocytes enclosed either singly or in masses between the other elements. Sometimes membranes of both types occur in the intestinal contents those of an epithelial character and those consisting of the above-mentioned non-epithelial elements." [Trans.]

Describing the gross appearances of the mucosa of the small intestine in cholera victims having succumbed early, Koch (1884) referred to com paratively few instances in which this membrane showed no marked changes, being merely somewhat swollen and less transparent, while the solitary follicles and Peyer s plaques were prominent. Though generally speaking the mucosa of the small intestines showed more or less intense reddening, sometimes this congestion was less marked or present only in spots. Instances were met with in which merely rims of congestion were visible round the follicles and Peyer s plaques—appearances which Koch considered particularly characteristic of cholera.

The observations of some of the subsequent workers, particularly those of Stoerk, indicated that the congestion of the mucosa of the small intestine met with in victims succumbing in the early stages of cholera, though apt to vary in degree and extent, was as a rule more marked than Koch had described Stoerk stressed however that the characteristic pink red colouration of the intestinal mucosa was fully manifest only when dissections were made within a few hours after death

Continuing his apt description Stoerk stated that

hand in hand with the reddening of the mucosa goes on the one side the constantly present but not always macroscopically recognizable hypersecretion of mucus, and on the other side the on the whole more frequent occurrence of mucosa haemorrhages both findings which I am inclined to consider as sequelae of the peculiar and almost specific hyperaemia " [Trans.]

In Stoerk s experience the mucous secretion became macroscopically manifest (*a*) more frequently in the form of flat, sharply circumscribed whitish layers, which became easily detached and (*b*) more rarely in the form of thicker tenaciously adherent masses with a rounded surface which mainly filled the depths between the intestinal folds, but could become more or less protuberant

General agreement exists that the appearances of the mucosa of the small intestines in cholera victims succumbing later in the disease are apt to show

Typically the small intestines of cholera victims succumbing early are brimful (*schwappend gefüllt*) with fluid the quantity of which may amount not rarely even to 3-4 litres (Liebermeister) To judge from the rather divergent statements made by different observers the character of this fluid may vary considerably Thus it has been maintained (see Kolle & Schürmann 1912) that

"in quite acute cases the intestinal contents consist of a slightly reddish fluid, in which numerous jelly-like pale-red lumps of mucus float, so that the masses are not dissimilar in aspect to coarsely minced meat extracted with a copious amount of water" [Trans.]

As noted for instance by Fraenkel (1892) sometimes, owing to the admixture of blood associated with an absence of flocculi the intestinal contents are of a reddish colour resembling that of diluted wine Further as stated initially by Koch (1884) and also recorded by other workers, e.g. by Fraenkel (1892), the intestinal contents found in cholera victims, while colourless may have the consistency of a thin gruel (*Mehlsuppe*) instead of that of rice water

Nevertheless, it has been maintained by other workers that the presence of rice water like intestinal contents identical in aspect with the typical cholera stools was the most constant and, at the same time, the most specific sign to be found in the dead bodies of early cholera victims. As claimed by Sticker (1912) Cruveilhier was the first who stressed the diagnostic importance of the *liquide cholérique* (i e., of the fluid intestinal contents) long before the discovery of *V cholerae* in his classical work on human morbid anatomy However a directly opposite opinion was expressed by Fraenkel (1892) who stressed that even in cholera victims succumbing early there was considerable variation in the aspect of the intestinal contents which could resemble thin gruel rather than rice water or be more or less haemorrhagic without admixture of flocculi. Still while one must fully agree with the contention of the last worker that bacteriological examinations are indispensable for the final post mortem diagnosis of cholera, one may claim that the presence of typical rice water like contents in the small intestines which generally appears to be more frequent than Koch s and Fraenkel s observations indicated, is of presumptive importance, particularly at the time of established cholera outbreaks.

It deserves attention that large amounts of typical intestinal contents may be found *post mortem* in the case of cholera victims who because they showed no diarrhoea had been diagnosed as suffering from cholera sicca —a form of the disease which, besides being as a rule infrequent (see the experiences in India quoted by Rogers, 1921) thus seems to be a clinical syndrome rather than a pathological entity

Giving a masterly detailed description of the contents found in the small intestines of early cholera victims, Stoerk stated the following

sible to deny that the desquamation takes place already during the life of the sufferers. This common sense view was held by Stoerk (1916) who concluded from his extensive and most careful studies that

"in accordance with the so frequent appearance of epithelial membranes in the cholera stools there can be no doubt as to the frequent occurrence of an intravital detachment of the epithelium" [Trans.]

At the same time however Stoerk maintained with great reason that a postmortal detachment of the intestinal epithelium not only took place as well but was probably more frequent. A similar view had been advocated already by Macleod (1910) who pointed out that "the amount of epithelial cells found in the [intestinal] contents after death exceeds that discovered in the stools during life"

While admitting the occurrence of an intravital epithelial desquamation Stoerk strenuously opposed the postulation of Fraenkel that the congestion of the intestinal mucosa represented a reaction to this process He maintained, on the contrary that in cholera an initial dilatation of the intestinal vessels was followed by an extravasation of fluid which in turn led frequently to an often extensive detachment of the epithelium from its basal membrane not followed by desquamation A concomitant or subsequent emigration of erythrocytes led to the formation of usually small haemorrhages in the mucosa of the ileum or sometimes of the whole small intestine which were situated mainly on the tops of the mucosa folds, but sometimes were seen to surround solitary follicles or to cover Peyer's plaques. Occasionally one could note the presence of more conspicuous haematomata under an intact epithelium.

The constant and rather characteristic presence of a subepithelial oedema in the small intestine of cholera victims was also noted by Goodpasture (1923) and more recently by Banerjee (1939) and Chatterjee (1939a)

Both Deycke (1892) and Stoerk (1916) concluded from their histological studies that the occurrence of necrotic changes in the intestinal mucosa of cholera victims was variable in frequency as well as in extent. Deycke maintained in this connexion that a coagulation necrosis involving either whole villi or only their tops, could appear in different stages of the disease being sometimes met with in victims who had succumbed within 24 hours while apt to be absent in those who had survived for 2 3 days.

Stoerk stressed that necrosis though frequently was by no means invariably present in detached or desquamated groups of the intestinal epithelia and upheld, therefore that

"one cannot by any means establish a closer relationship between necrosis and epithelial detachment both can appear quite independently of each other Apparently their common factor is only their causation through the cholera toxin." [Trans.]

Though the contrary has been asserted by a few workers more recently by Banerjee (1941) several other observers (see for example Deycke 1892

a most marked variance As aptly described by Simmonds (1892b) one may find in some of these instances

"extensive haemorrhages, specially on the top of the mucosa folds, in others marked swelling of the follicles and plaques In some cases the follicles are prominent in the form of pink red nodules, in others they are barely visible. Sometimes the mucosa is pale and smooth, in other cases lustreless and cloudy or covered with bran-like deposits. In some cases the mucosa shows an extensive dark-red colouration similar to that of an intestinal loop strangulated in a hernia. In the latter cases the intestinal contents show a dirty brownish red colour and have an offensive smell, whereas as a rule after the attack the intestinal contents of the jejunum possess a more egg-yolk-like colour those of the ileum as a rule a spinach-like colour and the large intestines are filled with yellowish-brown fluid and pap-like faeces" [Trans.]

Further, Simmonds continued, one was apt to find in the victims succumbing after an illness of three or more days in the small as well as in the large intestines necroses which localized particularly on the tops of the mucosa folds, occurred in different form and size as well as in varying numbers. If illness lasted longer the necrotic eschars were apt to become detached and flat ulcers with sharply defined margins could then take their place

The histological alterations observable in the small intestines of cholera victims have been the subject not only of numerous studies but also of much debate

One of the principal points at issue was the question whether the often extensive desquamation of the mucosa epithelium noted at autopsy was an intravital phenomenon or the result of a postmortal process. The former view which had been advocated already by Virchow (see his *Gesammelte Abhandlungen*, 1879) was also supported by the workers performing numerous autopsies during the 1892 cholera outbreak at Hamburg. Fraenkel (1893) one of these pathologists, maintained that in cholera the mucosa of the small intestine became "flayed" (*geschunden*) owing to a wholesale loss of its epithelium, and made this loss responsible for the marked congestion of the intestinal mucosa manifest in victims who succumbed in the early stages of the disease Deycke (1892) stressing that epithelial desquamation was observable in the small intestines of cholera victims dissected within a short time after death (in one instance after one hour) also felt certain that this process was of an intravital nature

The contrary view advocated by Cohnheim (1889 90) and some other early workers was more recently upheld by Goodpasture (1923) Since however this worker based his conclusions upon a detailed study of only 3 cholera victims, 2 of whom had succumbed late in the disease on the 6th and 9th days respectively when a regeneration of the originally desquamated epithelium was apt to have taken place, not much reliance can be placed on his contentions. Indeed, since desquamated epithelia are found invariably in the rice-water like evacuations of cholera patients, it is impos

Mesenteric lymph-nodes

Though some observers who had access to a large material like Crowell (1914) failed to detect lesions in the mesenteric lymph nodes of cholera victims most workers found these nodes to be usually enlarged and slightly or moderately congested Liebermeister (1896) noted that these alterations were most conspicuous in the lymph nodes in the region of the lower part of the small intestine while Sticker (1912) found them pronounced only when corresponding alterations were present in the plaques of Peyer

Kubo & Yuan (1933) noted in the 3 cholera victims dissected by them the occurrence of catarrhal changes in the sinuses and of degenerative processes in the germinative centres not only of the mesenteric, but of all lymph nodes

Liver

To judge from the rather discrepant descriptions of the different observers, the findings in the liver of cholera victims were apt to show considerable variation

Both Simmonds (1892b) and Fraenkel (1893) recorded that in their numerous autopsies they had never noted any gross changes in the liver Fraenkel asserted that histological changes were absent as well while Simmonds referred to the rare occurrence of what was evidently a parenchymatous degeneration of the liver cells.

Sticker (1912) and Crowell (1914) noted that in the victims who succumbed in the early stages of cholera the liver was not enlarged or —owing to the loss of fluid—even reduced in size (Crowell) but was rich in blood and consequently dark in colour The presence of venous congestion was also recorded by Macleod (1910) Liebermeister (1896) stated on the contrary, that in the victims succumbing early the liver was anaemic, flabby and fragile—appearances considered by Sticker as characteristic of cholera victims having succumbed later

It is certain that, in contrast to the statements of Simmonds and Fraenkel, often signs of cloudy swelling, and more rarely moderate degrees of fatty degeneration are met with in the liver of cholera victims (Liebermeister 1896 Crowell, 1914 Stoerk, 1916)

Ciaccia (1914) laid great stress upon the frequent occurrence of focal fatty changes in the liver of cholera victims, which were present particularly in the left lobe and near the falciform ligament and were macroscopically manifest by the presence of straw-coloured spots with a diameter varying from 0 5 cm to 3-4 cm, while the liver in general had a cherry red colour Certainly such appearances which never seem to have been noted by other observers,[1] are not pathognomonic for cholera, since the present writer saw them repeatedly in plague victims

[1] El-Ramli (1948) referred to one victim in whom histological examination of the liver revealed the presence of necrotic foci, generally distributed centrally and sometimes in the periphery of the lobules around the portal tracts" Apparently however *Schistosoma* ova had been found in the latter

Stoerk, 1916 and Chatterjee 1939a) stressed that in cholera cases not complicated by a secondary infection, regardless of whether necrosis had set in or not, there was never any evidence of an inflammatory round cell infiltration in the intestines

The evidence regarding the presence and arrangement of cholera vibrios in the histological sections made from the intestines will be dealt with in the following section of this chapter

Large Intestine

Dealing with the gross appearances of the large intestine in cholera victims who succumbed early Sticker (1912) stated that

"the large intestine specially the rectum, is as a rule empty narrow and contracted its mucosa shows no marked alterations. In contrast to the intensively reddened mucosa of the small intestine above the ileocoecal valve, it appears in the coecum already pale only in exceptional cases the hyperaemia continues to be present in the initial portions of the large intestine." [Trans.]

In the experience of many other observers as well usually no conspicuous changes were found in the large intestines, though occasionally besides congestion of the mucosa and of the usually pale serosa, the presence of extensive ecchymoses of the mucous membrane has been observed (Rogers, 1921)

It is noteworthy however that the findings of Stoerk (1916) did not tally with the descriptions recorded above He noted that the large intestine of cholera victims could be filled with fluid contents identical in character with those of the small intestines but that in other cases partly or even quite solid faecal masses could be present in the large intestines in spite of the presence of typical cholera evacuations in the small intestine A congestion of the mucosa throughout the large intestine could be met with and could be as intense as in the small intestine Its presence was usually associated with that of an oedematous swelling of the mucosa and submucosa, which produced a marked thickening of the intestinal walls.

While admitting that mucosa haemorrhages were definitely less frequent in the large than in the small intestines, Stoerk drew attention to an association of these haemorrhages with microscopically demonstrable mucosa necroses, which were apt to give rise to the formation of shallow roundish ulcers.

The histological findings in the mucosa of the large intestines showed in Stoerk s experience a marked analogy with those manifest in the small intestines of cholera victims except that there was no evidence of large scale epithelial detachment. Extensive mucosal necroses were often microscopically manifest in victims dissected soon after death and were apparently caused by an occlusion of the small blood vessels of the areas concerned through blood coagula or fibrinous thrombi, the formation of which was due presumably to an action of the cholera toxin

As summarized by Sticker several of the early workers had already ascribed the absence of a bile outflow in the early stages of cholera to nervous mechanisms such as a cramp of the sphincters of the biliary ducts or spasmodic contractions of the ductus choledochus or duodenum or finally a bile stasis conditioned by a general weakening of the reflex functions

The most likely explanation—that a nervous mechanism was at work—has been accepted also by modern observers like Stoerk (1916) and Chatterjee (1939b) Stoerk stated in this connexion that he had been unable

" to establish an evident relation between the absence or the presence of bile pigment in the stools and alterations of the gall-bladder or a possible relation to the degree of parenchymatous damage in the liver Perhaps this is a rather complicated process I am inclined to think of the possibility that besides a toxic impairment of the liver secretion a role could be played by a disturbance of the reflectory processes regulating the influx of the bile through the papilla into the small intestine " [Trans]

As will be gathered from the statements made above, in cholera victims succumbing early the gall bladder was almost invariably found to be filled with more or less normally coloured bile which however as stressed by several observers often showed an abnormally thick consistency In the case of victims who died in the later stages of the disease on the contrary the gall bladder was usually found to contain thin and lighter coloured occasionally even water-clear bile (Simmonds, 1892b Liebermeister 1896 Macleod, 1910)

Marked gross alterations in the gall bladder of cholera victims though on the whole not frequent, have been recorded by a considerable number of workers—first apparently by Pirogoff who as quoted in the summaries of Kulescha (1910) and of Coulter (1915) referred in a monograph on the morbid anatomy of cholera published in 1850 to 2 instances in which a diphtheric cholecystitis had been found in cholera victims one of whom had succumbed to a generalized peritonitis due to a perforation of the gall bladder

Important further observations on this point may be summarized as follows.

Simmonds (1892) performing more than 300 cholera autopsies, noted once the presence of necrosis of the gall bladder mucosa and 4 times that of cholecystitis characterized by swelling and congestion of the mucosa and also by admixture of pus to the bile Apparently however in these instances the inflammatory process was due to a secondary invasion of cocci instead of being caused by *V cholerae*

Kulescha (1909) described the case of a male succumbing about a month after he had been attacked by cholera who showed at autopsy signs of (*a*) acute enteritis with necroses, specially of the colon, the perforation of which had led to peritonitis (*b*) septicopyaemia with endocarditis and (*c*) purulent cholangitis. Histological examination proved that the purulent inflammation of the intrahepatic bile passages had led to round-cell infiltrations and

Reference to further alterations occasionally observed in the liver of cholera victims, which were secondary to affections of the gall-bladder and the biliary ducts will be made in the following paragraph

Gall-bladder and biliary passages

The conditions met with at autopsy in the gall-bladder of cholera victims have been the subject of many studies. As summarized by Sticker (1912) as long ago as the 1817 outbreak in India, British observers had been struck, when dissecting victims who had succumbed early, to find contrasting with the acholic intestinal contents, a superabundance of normally coloured bile in the gall-bladder This paradoxical phenomenon also attracted the attention of all subsequent workers who tried to account for it in various ways

It was occasionally claimed that a mechanical obstruction, created by the presence of mucus plugs occluding the entrance of the common bile duct into the duodenum or through congestion and swelling of the mucosa at this spot (see Rogers, 1921) prevented the outflow of the bile from the gall bladder There can be no doubt, however that such mechanical obstructions are extremely rare, most observers finding that almost invariably a slight pressure exerted at autopsy on the gall-bladder sufficed to start an outflow of the bile into the duodenum.

As pointed out by Sticker (1912) in the opinion of several early workers

"the scarcity of bile in the dejecta was due merely to a high dilution of the intestinal contents through the tissue transudates, since even in dead bodies succumbing in the algid stage very often bile was found in the duodenum and traces of it were sometimes still present in the lower part of the small intestines." [Trans.]

More recently Kubo & Yuan (1933) expressed the belief that in addition to its subnormal secretion the bile's dilution in the intestinal contents accounted for the acholic condition of the cholera evacuations

Being an ardent supporter of the views of Emmerich (see Emmerich & Tsuboi, 1893) who considered the manifestations of cholera to be due to nitrite poisoning, Sticker maintained that there existed no real acholia of the intestinal contents and faeces, but that the bile was decolorized in the intestines through the nitrous acid formed there by *V cholerae* He mentioned in support of this postulation that a few observers had found the presence of cholera vibrios in the bile associated with a watery or milky aspect of the gall bladder contents However ample observations of later workers failed to confirm the frequency or even the occurrence of such coincidences Thus Greig (1913a) recording 81 instances in which cholera vibrios had been isolated from the gall-bladder of cholera victims never mentioned a watery or milky aspect of their bile. More important still, the postulation of Emmerich that cholera was the result of nitrite poisoning has been categorically rejected by almost all other observers.

examinations of the bile had been made It was noteworthy however that in about 4% (10 cases) of this series also gross signs of cholecystitis had been detected

Giving at the same time a description of the gall bladder alterations met with in bile positive cholecystitis cases, Greig stated that under these circumstances frequently a thickening of the wall of the gall bladder as well as signs of congestion of the mucosa and sometimes also the submucosa were grossly manifest The histological changes consisted of desquamation of the mucosa epithelium, infiltration of the submucosa with polynuclear and mononuclear leucocytes, in some cases also of haemorrhages or evidence of the recent formation of blood vessels and the presence of foci of round cells in the serosa The penetration of *V cholerae* into the tissues of the gall bladder wall was confirmed with the aid of cultivation of the organisms

Desquamation of the epithelium and round-cell infiltration of the submucosa associated with the presence of cholera vibrios were also found in sections made from the hepatic, cystic, and common bile-ducts. Moreover Greig stated that

"in addition to the pathological changes observed in the gall-bladder and the cystic, hepatic and common bile ducts in cases of cholera, there are alterations of an inflammatory nature in connection with the biliary passages in the liver itself and even between the columns of liver cells. The presence of true cholera vibrios was determined in the liver tissue by cultivation as well as by examination of sections."

Crowell (1914) noting only 3 instances of marked gall bladder inflammation in his 92 cholera autopsies, considered the presence of this condition or of gall bladder hydrops as rare. Similarly Coulter (1915) summarizing the results of 635 cholera autopsies in Manila (presumably including those of Crowell) stated that an inflammation of the gall bladder was met with but 12 times, an inflammation of the bile ducts once. The former number probably included the 3 instances described by Schoebl (1915) to whom Coulter stated that he had sent 39 tied-off gall bladders Schoebl succeeded in isolating cholera vibrios from the bile in 17 of these organs, but met with macroscopic lesions in only three Twice a hydrops of the gall bladder was found to be present, characterized by distension of the organ which was filled with a mucous fluid of light-amber colour with a flaky sediment, a milky colour becoming apparent upon stirring. In the third case the gall bladder was small showing apparently thickened walls and rather dark contents Histological examination showed epithelial desquamation as well as congestion and round-cell infiltration of the mucosa. Apart from desquamation of the epithelium identical changes were found in the cystic duct.

The significance of the positive bacteriological findings in the gall bladder of cholera victims will be discussed in the following section of this chapter

necrosis in the adjacent parts of the liver in which also metastatic abscesses not connected with the bile passages were found. The generalized infection was no doubt due to a secondary invasion mainly of *E coli* and diplococci, but Kulescha succeeded in isolating cholera vibrios from the pus in the bile passages He added that in the 109 cholera victims he had dissected, the causative organisms had been detected in the bile in 49 instances and that in a considerable number of these instances signs of cholecystitis had been found—invariably in cases of longer duration (5-6 days)

In 1910, Kulescha, reporting upon a total of 430 cholera autopsies, stated that he had found signs of acute, usually purulent cholecystitis in 42 instances, i e. in nearly 10% half of these victims had succumbed within the first week, most of the others within the second week of illness. In only 8 of these cases did the gall bladder contain a colourless thick fluid Microscopically the mucosa of the gall bladder was found to be denuded of its epithelium and infiltrated with round cells, while the submucosa showed besides round-cell infiltration, signs of marked congestion with some blood extravasation.

In four of these cases gross and histological signs of an acute chol angitis were found. This process led invariably to an involvement of the adjacent liver tissue so that Kulescha felt entitled to class 3 of these instances as purulent biliary hepatitis, while he considered the fourth a case of hepatic biliary cirrhosis. Cholera vibrios were detected in all 4 instances by bacteriological and histological methods, the organisms seen in the sections showing signs of involution.

Kulescha also referred to the extraordinary case of a woman who admitted with signs of cholera and marked jaundice, continued to harbour the causative organisms in her stools for 57 days 7 months later she was again hospitalized with an enlarged and painful liver but succumbed only after a further period of 4 months. At autopsy signs of cholangitis with marked biliary stasis were found *V cholerae* though absent from the intestines, could still be recovered from the contents of the intrahepatic bile ducts. However as Kulescha added, the possibility that this woman had been reinfected with *V cholerae* before she was hospitalized the second time could not be excluded

Further exhaustive studies on the subject presently under review were made by Greig. Following up a preliminary note on the occurrence of cholera vibrios in the biliary passages published in 1912, he reported in 1913 on 271 autopsies of cholera victims, in 81 of whom the causative organisms were isolated from the bile and in 12 of whom (4 4%) gross signs of cholecystitis were found Histological examinations demonstrated the presence of *V cholerae* not only on the mucosa but in this as well as in the submucosa

In a subsequent communication Greig (1914b) referred to a further series of 235 autopsies of cholera victims in whom no bacteriological

examinations of the bile had been made It was noteworthy, however that in about 4% (10 cases) of this series also gross signs of cholecystitis had been detected

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Spleen

Many observers were in agreement with the findings described in the spleen of cholera victims by Liebermeister (1896), who summarized that (*a*) in those who succumbed early the organ was small and flabby with a wrinkled surface, on section dry and rather anaemic, while (*b*) in victims having succumbed later the spleen was sometimes enlarged, usually richer in blood and of normal turgor and occasionally showed the presence of infarctions. Simmonds (1892b) maintained in this connexion that spleen enlargement was almost exclusively met with in instances where concomitant processes or their sequelae were present. Similarly Fraenkel (1893) stated that he had met with a large spleen but once in the case of a secondary infection with streptococci Both he and Simmonds noted the presence of pulpa haemorrhages in a minority of the victims

It is noteworthy however that in the experience of Stoerk (1916)

" the spleen showed in uncomplicated cases a somewhat increased consistency and I do not believe I have seen a case in which it did not seem at least a little enlarged. A considerable enlargement, however was never observed, at least not in cases where a spleen enlargement through complicating diseases could be excluded. The almost invariably present hyperaemia due to cardiac insufficiency appeared to account for the consistency and the enlargement of the organ." [Trans.]

According to Stoerk, the histological alterations in the spleen were rather variable. As a rule the pulpa was rather rich in cells, while the follicles were somewhat enlarged

Rogers (1921) and Chatterjee (1939a) also referred to congestion of the spleen and to hyperplasia of the Malpighian bodies, the latter observer moreover stating that the organ was definitely enlarged in a high proportion of his autopsies. One cannot help feeling that these two workers as well as Stoerk described the condition of the spleen in the later rather than in the early stages of cholera.

Bone-marrow

Fraenkel (1893) constantly found in the acute stage of cholera a marked haemorrhagic condition of the bone marrow which was sometimes diffuse, and in other cases manifested only in the form of foci (*herdweise*).

Similar observations were also made by Kulescha (1910) who, as summarized by Greig (1929), distinguished between 2 stages in the condition of the bone marrow in cholera victims namely (1) an erythroblastic reaction to the deficiency of oxygen in the blood and (2) a granulo-cellular hyperplasia, representing a reaction to the infection itself

Chatterjee, who had already dealt with the alterations of the bone marrow in cholera in an earlier article (1939a) summarized in 1947 his experiences when studying the bone-marrow of the humerus, femur and tibia in a total of 40 victims He found that macroscopically the bone-marrow

"may present a red appearance in cases in which the reactive hyperplasia is marked. In others pink areas interspersed with white or fatty marrow present the usual picture. The general consistency is more solid than that of inactive fatty marrow and the specimens are sectioned more easily."

Through histological examinations it was established that

"(1) There is an acute dilatation and engorgement of the normally collapsed system of capillaries of the bone marrow in cholera.

"(2) These capillary changes are more marked than in any other organ in cholera and might at least in some way partially explain the condition of great shock in the disease.

"(3) Owing to the widening of the above capillaries it is possible to study their openings into the venous sinusoids of the bone marrow

"(4) The sinusoids are also distended

"(5) There is an increase in the number of eosinophils, and a variable amount of leucoblastic reaction of the marrow

"(6) Small lymphatic nodules have been observed in some cases."

Suprarenals

The condition of the suprarenals in human cholera victims seems to have been studied by only a few workers. Sticker (1912) briefly noted that in victims succumbing early the suprarenals were usually found to be dry and anaemic. Banerjee (1939) who paid attention to these organs in view of the possible importance of an adrenal insufficiency in the production of the cholera syndrome described degenerative changes in the cortex of the suprarenals. According to De Sengupta & Ganguli (1955) Chatterjee (1939b) studying the suprarenals of some cholera victims established the presence of a congestion of the boundary zone which he thought to be related to the condition of extreme shock in the first stage of the disease

Quite recently De and co-workers (1955) examining the suprarenals of 11 cholera victims, who had succumbed within 48 hours in the stage of shock, found that a depletion of the cortical lipoids had taken place to a varying degree and postulated that this evidence suggested

"an active role of the suprarenal cortex for enhanced synthesis of its hormones in response to the stress of dehydration, shock and anoxia of cholera"

In view of these divergent statements one must fully agree with De and colleagues that there is need for further inquiries into the functional state of the pituitary-suprarenal system in cholera

Kidneys

As stated in a classical summary by Leyden (1893) the early observers, though vastly differing in the opinions they held in regard to the nature of the kidney lesions in cholera, were fairly unanimous when describing the alterations actually met with in the victims Referring to the macroscopic findings, Leyden stated that

all observers agree that the cholera kidneys in rapidly succumbing cases show hardly any external changes In protracted cases they are enlarged, hyperaemic with small

haemorrhages and sometimes show cuniform infarctions. From the papillae one may express a turbid fluid rich in casts and in cells. Microscopically it is particularly striking to find cloudy swelling and partly fatty degeneration of the epithelia. The interstitial tissues as well as the capsules of Malpighi appear to be free the cortical tubules are often enlarged and filled with a granular fibrinous or cellular material, the collecting tubes of the papillae are thoroughly plugged with casts. To these descriptions E. Klebs [1887] added the important finding of a coagulation necrosis ...

"All observers likewise agree that this affection of the kidney is of short duration and apparently never passes into a chronic stage." [Trans]

As Leyden added he had been able to confirm the presence of an extensive coagulation necrosis of the epithelia in one of the four kidneys examined by him in 2 other instances the epithelia of the renal tubules were intact, but there were within their lumen as well as within the capsules of Malpighi more or less numerous cells showing the features of necrosis.

It is interesting to compare this early description which was based in part on the results of the pioneer investigations by Reinhardt & Leubuscher (1849) with the most recent findings made by De Sengupta & Chanda (1954) in the kidneys of (*a*) 14 cholera victims who had succumbed in the state of shock (*b*) 10 who had died in the stage of reaction and (*c*) 8 individuals, in whom uraemia had supervened As noted by De and co-authors, in the first group

"The stellate vessels beneath the capsule appeared injected, while the intervening areas looked pale. The cut surface of the kidney showed evidence of pallor of the cortex with congestion of the medulla. The interlobular vessels stood out conspicuously against the pale background."

Histological examinations showed the presence of a cortical ischaemia, engorgement of the medullary vessels, and also of oedema in the connective tissue separating the medullary tubules Parenchymatous changes included some degree of cloudy swelling of the epithelium in the cortical tubules, in two instances fatty and necrotic changes in the tubules.

In the stage of reaction no evidence of cortical change or of vascular disturbance was found in the kidneys, the cut surface of which exhibited a uniform hue of both the cortex and the medulla. The tubular epithelium showed cloudy swelling in some cases only while fatty changes of the tubular epithelium were noted exclusively in one kidney showing evidence of tuberculous lesions

As summarized by De and his colleagues

"The kidneys of postcholeric uremia show signs of cortical ischemia and of medullary congestion which are more marked than in the stage of shock. Necrosis and fatty change of the cortical tubules and thickening and splitting of the glomerular basement membrane are constant features. Besides, evidence of repair and of total necrosis of cortical substance may be found in some specimens. The medulla is uniformly congested and is free from any sign of damage."

It is of great interest to note that the valuable investigations of De and colleagues confirmed the early observations made by Klebs and by Leyden

in regard to the occurrence of necrotic changes in the cholera kidneys As shown below, the views held by these recent workers regarding the nature of the kidney alterations met with in cholera victims also showed a quite close agreement with Leyden s postulations

As can be gathered from the classical summary of this author, a few of the early observers were inclined to classify the morbid process developing in the kidney of cholera sufferers in the category of nephritic affections French (1851) for instance, dealing with it in his monograph on Bright s disease In marked contrast to this view Griesinger (1857) felt convinced that the renal manifestations characteristic of cholera were not due to a local inflammatory process, but were the indirect result of the enormous dehydration and the marked lowering of the arterial pressure becoming apparent in severe attacks of the disease This idea was vigorously supported by Bartels (1875) who aptly spoke in this connexion of an ischaemia of the kidneys.

Though Griesinger s views were at first widely accepted, they were set aside when as Leyden excellently put it owing to recent discoveries including that of *V cholerae*

" the experimental pathology lost its importance [*blasste ab*] and the bacteriology became the focus of interest and of concepts " [Trans.]

Accordingly it was advocated by several observers, e.g., by Klebs (1887) Aufrecht (1892) Fraenkel & Simmonds (1892) and Fraenkel (1893) that the renal manifestations in cholera sufferers were due to an action of the toxin of *V cholerae* on the kidneys However, Leyden (1893) took a determined stand against the claims of Klebs and Aufrecht that Griesinger s view was erroneous because the kidneys even of cholera victims having succumbed early were apt to be hyperaemic Leyden pointed out that the process in question was not one of a stoppage of the blood supply but one of a markedly lowered blood pressure and that consequently remnants of blood, capable of producing a veinous congestion, could continue to be present in the renal vessels Emphasizing that (*a*) the alterations observed in the kidneys of cholera sufferers were altogether different from the nephritic processes caused by bacterial toxins in other infectious diseases and (*b*) in contrast to what was observed in cholera, the acute nephritic processes of other infectious diseases could pass over into a chronic state and even result in renal atrophy Leyden concluded that

owing to its rapid course the cholera nephritis rather shows a certain analogy with the nephritis of pregnancy or eclampsia, ascribed hitherto to circulatory disturbances during pregnancy which became quickly abolished after parturition, thus accounting for the usually rapid course of the process " [Trans.]

It is of great interest to note that views closely similar to this quite forgotten statement of Leyden have been recently expressed by several observers. Thus Maegraith and colleagues (1945) quoting Rogers (1921)

Chatterjee (1941), and Tomb (1942) maintained that cholera was one of quite numerous conditions in which a syndrome of renal "anoxia" secondary to peripheral vascular failure accounted for the manifestations on the part of the kidneys

On account of these statements and their own observations, De, Sengupta & Chanda (1954) reached the conclusion that

"Reduction of renal blood flow aided by some degree of cortical vasospasm is thought to bring about a complete cessation of urinary secretion in the stage of shock. These factors are, however usually incapable of causing any morphological renal damage, although functional damage is indicated by the occurrence of albuminuria and hematuria in the following stage. In a minority of cases, the occurrence of hemoglobinemia is a possible factor in intensifying the renal vasospasm and in rendering more complete the cortical ischemia, which is responsible for serious structural damage leading to post choleric uremia."

As far as the present writer is entitled to judge, the above postulations offer a far more satisfactory explanation for the genesis of the renal manifestations in cholera than the assumption of a direct action of the cholera toxin on the kidneys In this connexion, De and colleagues pointed out with much reason that, whereas in human cholera victims the ischaemia and the subsequent renal lesions showed a cortical localization, there was no experimental evidence to claim a selective action of the cholera toxin on the cortical tubules alone Agreement with these views has recently been expressed by Banerjee & Ghosh (1957), who were inclined to ascribe the marked diminution of the glomerular filtration rate and the renal plasma flow found by them after restoration of the urine flow in cholera patients as well as in sufferers from gastro-enteritis "to renal anoxia causing temporary diminution in blood flow through the cortex of the kidney and not to any specific toxins of the disease"

Urinary passages and urinary bladder

The appearances met with in the urinary passages and the urinary bladder of cholera victims depend upon the stage in which death took place As long as anuria had been manifest, the calices and the pelvis of the kidneys contain minimal amounts of a thick mucous fluid in which desquamated epithelia and casts are present. The urinary bladder is contracted and, if not practically empty contains a little of the same fluid or a few millilitres of turbid urine in which besides numerous casts erythrocytes and, according to Sticker also some leucocytes may be found. Crowell (1914) recorded in this respect the following figures

Condition of the urinary bladder	*Number of observations*	*Percentage who had received saline infusions*
Contracted and empty	60	11.6
Containing 2-3 ml urine	18	77.0
Unknown	14	—
Total number of autopsies	92	

In the later stages of cholera when urine secretion has been restored the urinary bladder is often found to be quite full so that as justly stated by Simmonds (1892b) the reappearance of the urinary flow *per se* does not invariably indicate a favourable prognosis

As generally agreed the presence of haemorrhages on the mucosa of the urinary passages and bladder is quite frequent Simmonds, who seems to have paid special attention to this point found that the bladder hae morrhages could become so numerous and extensive as to lead to an admixture of blood to the urine

The presence of cystitis or of diphtheritic or gangrenous changes in the mucosa of the urinary bladder in the later stages of cholera has been recorded by some observers e g. by Simmonds (1892b) and Deycke (1893)

Genital apparatus

While no conspicuous changes have been found in males, marked and interesting alterations have been recorded quite often in the uterus and the vagina and sometimes also in the ovaries or ovarian tubes of female cholera victims.

As can be gathered from an article published in 1872 by Slavjanski and a later summary by Klautsch (1894) these alterations had already attracted the attention of several early observers some of whom noting the conditions they had met with in the uteri and ovaries, spoke of the presence of a "pseudo-menstrual" state in cholera victims Slavjanski himself claimed that the process observable in the uteri of non pregnant subjects was of an inflammatory nature and spoke likewise of the presence of an "endometritis decidualis haemorrhagica" in pregnant cholera victims

After the discovery of *V cholerae* the alterations of the genital apparatus in female cholera victims received particular attention by Tipjakoff (1892) Simmonds (1892b) Deycke (1893) and Klautsch (1894)

Tipjakoff noted that in non pregnant females who fell victims to cholera invariably the serosa and more markedly still the endometrium of the uterus were hyperaemic, while small haemorrhages were present in the uterine tissues. In some instances large coagula were found in the cavity of the uterus, twice a haemosalpinx. Histologically, there was no evidence of a round-cell infiltration in the uterus.

In the 6 pregnant cholera victims seen by this worker who succumbed after they had abortions or miscarriages conditions similar to those de scribed above were found in the serosa of the uterus but the tissues of this organ in general appeared to be anaemic and large coagula were found to be present on the site of attachment of the placenta

Simmonds referred to the very frequent occurrence of haemorrhages on the endometrium, which occasionally was found to be markedly swollen In about a third of the victims who succumbed in the later stages of cholera the presence of superficial necrotic areas in the vagina was noted and in two

instances an extensive necrotic process involving the adjacent tissues was found. In the ovaries, haemorrhages were occasionally seen to be present.

Deycke recorded the occurrence of marked alterations in the uterus of 65% of the 170 non-pregnant cholera victims seen by him. Besides more or less copious haemorrhages into the uterine cavity he regularly noted even in the case of victims succumbing early the presence of either superficial or more deeply penetrating haemorrhagic infiltrations of the endometrium Histologically evidence of a haemorrhagic infarction of the endometrium was found together with signs of marked congestion of the parts of the endometrium not infarcted and the muscular layers of the uterus. Moreover, not rarely signs of a superficial coagulation necrosis of the endometrium were met with and necrosis was found to be extensive in one case where a secondary invasion of streptococci had taken place Superficial necrotic areas and once an ulcerative process were found in the vagina, but these alterations seemed to be due to secondary infections.

Klautsch (1894) summarizing the results of an examination of the genitalia of 11 adult non pregnant cholera victims, maintained that these organs were in a condition of inflammation as well as of hyperaemia. Moreover haemorrhagic foci were found in (*a*) the uterus mucosa, (*b*) the old corpora lutea of the ovaries, and (*c*) the subserous tissue of the tubes Klautsch added that there also occurred the formation of small granules consisting of iron-containing pigment in these foci of extravasation.

Ample experience has shown that, though exceptions were not altogether rare as a rule cholera attacks of pregnant women led to an intra uterine death of the foetuses during the early stages of the disease followed by abortion or miscarriage

Referring to such observations, Tizzoni & Cattani (1888) stated that, while some of the early workers found no gross alterations in the foetuses of cholera victims, others described the occurrence of blood stained transudations in the serous cavities or of congestion of the small intestines which then contained instead of meconium a clear or greenish or even a rice-watery fluid.

Tizzoni & Cattani themselves examined the foetus of a cholera stricken woman who being in the 5th month of pregnancy had an abortion on the 4th day of illness but afterwards recovered. They found that the serous cavities of the foetus contained a sanguinolent fluid. The small intestine was congested and contained a soft mass of reddish colour Cholera vibrios could be cultivated not only from the intestinal contents but also from the heart blood and the serous cavities

Further observations were recorded by Segale (1913) who found in 3 stillborn children of sufferers attacked by cholera during advanced pregnancy (*a*) subpleural and subpericardial haemorrhages (*b*) marked degeneration of the liver and kidneys and (*c*) haemorrhages or even haematomata in the suprarenals. While cultures made from the intestine

and blood of these foetuses gave negative results the serum of 2 proved toxic for guinea pigs 0 25-ml amounts killing the animals in less than half an hour Segale maintained therefore that besides an asphyxia produced by the congestion of the maternal organs poisons elaborated by the mothers might have been responsible for the intra uterine death of these foetuses.

In contrast to these experiences, Simmonds (1892b) though able to make fairly ample observations in this respect, stated that he had never found in the foetuses of cholera victims any signs indicating either the presence of *V cholerae* or an action of its toxin Similarly Tipjakoff (1892) maintained that difficulties of gas exchange promoted by the occurrence of haemorrhages between the uterus wall and the placenta accounted for the intra uterine death of the foetuses of cholera stricken mothers Klautsch (1892) postulated in a well-documented article that the factors at work in this respect were (*a*) the dehydration of cholera victims which by leading to a lowering of the blood pressure and consequently to an insufficient intake of oxygen by *the maternal organs, also rendered the placental* respiration deficient (*b*) alterations in the foetal portion of the placenta in which *according to Slavjanski (1872) epithelial decay could take place* and (*c*) haemorrhages between the uterus wall and the placenta which as *observed by Tipjakoff and by Klautsch himself could cause a partial* detachment of the placenta Nevertheless while not believing in an intra *uterine infection with V cholerae Klautsch considered the possibility* that an action of the cholera toxin played a role in the death of foetuses of cholera victims *in utero* Schütz (1894) *in a lecture on the influence of* cholera on menstruation pregnancy birth and the puerperium, the text of which was available to the present writer *in the form of a summary only* was evidently inclined to consider this toxin responsible for the contractions of the uterus and for the haemorrhages from the uterine mucosa observed during attacks of the disease in pregnant as well as non pregnant patients

Distribution of the Causative Organisms in the Dead Bodies of Cholera Victims

Intestinal contents

Reporting in 1884 on the observations he had made in nearly 100 instances, Koch stated that he had invariably found the comma bacilli in the intestinal contents of cholera victims and added that

> the examination has not only shown that they [i e. the comma bacilli] are *present* but that their presence is always directly proportionate to the cholera process. For where the cholera process proper produces the most profound alterations in the intestine, namely in the lower segments of the small intestine, they were most numerous, becoming less numerous in the upper parts. In the most clear cases they were present in nearly pure culture, but became the less conspicuous the older the cases were and the more secondary alterations had taken place in the intestine " [Trans.]

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as will be discussed in the seventh chapter, the present aim of practical cholera laboratory diagnosis is a rapid isolation of *V. cholerae* for the purposes of serological identification no more stress is laid upon smear examination In fact besides giving by no means rarely negative results this method may prove misleading in view of the possible presence of cholera like vibrios in the intestinal contents

Intestinal walls

Describing his histological findings in the intestinal wall of cholera victims, which led to the discovery of *V. cholerae* Koch (1884) stated that the suspected bacteria

> "had partly penetrated into the tubular glands and partly had advanced between th epithelium and the basal membrane thus, as it were detaching the epithelium In other places it was seen that the organisms had penetrated also more deeply into the tissues. Further cases were found in which behind these bacteria, which had a peculiar size and aspect, so that one could distinguish them from other bacteria and could devote special attention to them different other bacteria penetrated into the tubular glands and the surrounding tissue Thus conditions were created similar to those in necrotic, diph theric alterations of the intestinal mucosa and in typhoid ulcers, where likewise subsequently other non-pathogenic germs penetrate into the tissue destroyed by pathogenic bacteria." [Trans.]

Koch illustrated these findings by a drawing showing an oblique section through a tubular gland of a cholera affected intestine in which numerous typical vibrios could be seen between the epithelium and the basal membrane besides some in the lumen as well as a few deeper in the intestinal tissue [1]

Babes (1885) summarized corresponding observations on the intestine in cholera victims by stating that the causative organisms were mainly assembled on the mucosa surface and under the epithelium, whereas

> "in the interior of the mucosa one sees the comma bacilli as if they had entered accidentally without a definite arrangement. Later on, when dysentery-like processes have replaced these alterations, there occurs a massive penetration of the bacilli into the deep layers, where they can form dense agglomerations these organisms are usually larger than the cholera bacilli. In rare cases one sees colonies of micrococci in the necrotic mucosa." [Trans.]

In contrast to these findings, Deycke (1892) maintained that even in cholera victims succumbing rapidly a penetration of the vibrios was invariably found to have taken place in some parts of the intestinal wall—partly more or less deeply into the necrotic tissue of the villi or still further into the submucosa, sometimes to points not far from the muscularis. An agglomeration of the vibrios in Lieberkühn s crypts was very frequent

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[1] A coloured reproduction of this drawing is attached to the article of Kolle & Schürmann (1912) and also to that of Kolle & Prigge (1928).

Besides thus admitting that the bacteriological findings made in the intestinal contents of cholera victims were apt to vary according to the stage of the disease Koch also stressed the necessity of resorting to cultural methods, since the demonstration of the comma bacilli in smears sufficed for a diagnosis "only in comparatively few cases" He also emphasized the importance of performing the autopsies as soon as possible after the death of the victims before putrefaction of the intestinal contents and walls had commenced.

Though the occurrence of the causative organisms in the intestinal contents of cholera victims was also investigated by some other early workers the first really exhaustive studies were made by the pathologists performing numerous autopsies during the 1892 Hamburg outbreak.

Simmonds (1892b) one of these observers, maintained that in cholera victims succumbing early the vibrios could invariably be demonstrated in smears from the intestinal contents, but often appeared to be present in surprisingly small numbers. Usually they were most conspicuous in smears made from the ileum contents, less numerous in the large intestine, still less in the jejunum, and least in the duodenum. However exceptions to this rule were found, the vibrios, while scanty in the ileum, being plentiful in the large intestine or even in the jejunum In contrast to these variable findings, cultivations from all parts of the intestine gave abundantly positive results.

While admitting that no definite rules could be laid down regarding the presence or absence of *V cholerae* in the intestinal contents of sufferers who had succumbed to the disease in different stages, Simmonds maintained that

"before the 6th day I have never missed the bacilli, between the 7th and the 12th days I still found them in more than half of the cases, after the 12th day but exceptionally" [Trans.]

Fraenkel (1892) though also recommending culture methods for the post mortem diagnosis of cholera, found that smear examinations were apt to give frankly positive results as long as the intestinal contents were of a typical appearance with numerous flocculi He stated in this connexion in his 1893 paper that in 536 autopsies performed on victims who succumbed in various stages of cholera, smear examination proved positive 442 times, while giving a negative result in 94 instances—almost exclusively in the case of victims who had died later than on the 4th day of illness. In a considerable number of these negative cases, culture tests as well failed to give a positive result.

Further experiences, to which reference will be made below while confirming that it is not invariably possible to isolate the causative organisms from the intestinal contents of cholera victims, have shown that bile cultivation forms an important additional means to obtain positive results. Since

apparent than real depending largely upon the usually but casual attention given to a bacteriological examination of the vomits It is in accord with this assumption that, while Simmonds (1892b) stated that he had found cholera vibrios in the stomach contents at only few of his autopsies Tizzoni & Cattani (1888) recorded such findings in 3 out of 5 cholera victims and Brüloff (1910) in 78% as against over 90% positive results obtained with the contents of the small intestines and 62% with the rectal contents.

Oesophagus

Sewastianoff (1910) stated without furnishing details that he had found cholera vibrios in the oesophagus of 2 out of 14 cholera victims How often he actually examined this organ is not clear

Gall-bladder and biliary passages

The occurrence of cholera vibrios in the gall-bladder and more rarely in the biliary passages of cholera victims has been demonstrated by a considerable number of observers first apparently in 1885 by Doyen Kelsch & Vaillard and Nicati & Rietsch

Important findings made in this respect may be summarized as follows

Nicati & Rietsch (1885) found *V cholerae* to be present in the bile of 4 out of 8 cholera victims—twice in the gall bladder and twice in the biliary passages.

Studying the action of the *bacille virgule* (comma bacillus) on the liver and pancreas of cholera victims Girode (1892) found in 14 out of 28 instances the organisms to be present in the gall bladder as well as in the bile ducts, and 6 times in pure culture Identically positive results were also obtained (*a*) in the case of a cholera victim who succumbed on the 18th day showing signs of cholecystitis and generalized angiocholitis with involvement of the adjacent liver tissue and (*b*) in a second victim succumbing to cholera after 13 days and showing at autopsy signs of a presumably pre-existent atrophic liver cirrhosis

As quoted by Sewastianoff (1910) Rekowsky (1892) found during the 1891 outbreak in St. Petersburg cholera vibrios in the gall-bladder of all 14 victims examined by him

Lesage & Macaigne (1893) established in the course of an interesting investigation that (*a*) cholera vibrios were absent from the gall bladder of 7 victims who having succumbed in the algid stage and having been dissected immediately showed the causative organisms in their intestines and (*b*) the vibrios were present in pure culture in the bile of 3 out of 18 victimes who having also died in the algid stage were autopsied after 4 hours A postmortal invasion by *E coli* was manifest in the other organs of these 3 dead bodies

According to the ample experiences of Stoerk (1916) the cholera vibrios were to be found almost exclusively above the surface of the epithelium, and mainly in the contents of the intestine

"but in large part immediately on the epithelium, as if affixed, or in the mucus of the crypts. In the first case (in the intestinal contents) they often form voluminous complexes, in the latter they appear as small and very small groups, less often more singly" [Trans.]

Stoerk's comment upon these findings was as follows

"In numerous descriptions it has been stated that—apart from their main occurrence on and above the mucosa surface—the cholera vibrios can be found also in the *depth* of the mucosa In contrast to this I must emphasize on account of my microscopic studies that, in sections of specimens preserved an adequately short time *post mortem* and in the absence of ulcerative alterations, I almost never saw certainly identifiable vibrios *below* the basal membrane of the intestinal epithelium. The occurrence in the lumen of the crypts is almost constant but I hardly ever encountered in freshly preserved specimens an arrangement of a clearly intravital origin between the loosened epithelium and the basal membrane of the kind illustrated, for instance, by R. Koch

"No doubt in many cases a considerable postmortal enrichment of the intestinal flora takes place since we do not possess a specific staining method for the cholera vibrios and their form is frequently quite uncharacteristic, endeavours to identify the vibrios in the maze of bacteria present in sections of specimens from not immediately dissected cases often give no satisfactory results. Identical difficulties may be encountered in the case of sections from freshly obtained post mortem specimens and I also cannot ascribe great value to a comparison of the sections with the smears made from the intestinal contents of one and the same case." [Trans.]

In view of these difficulties Stoerk concluded that

"much of what has been stated so far on account of histological findings regarding the occurrence of vibrios in the intestinal walls has to be accepted with considerable restrictions" [Trans.]

As will be further discussed below Goodpasture (1923) maintained, in agreement with the views of Stoerk, that the cholera vibrios remained mostly confined to the intestinal lumen

Stomach

Recording the results of his pioneer studies, Koch (1884) maintained that, though he had very frequently examined the vomits of cholera patients,

"cholera vibrios had been found in them but twice, and in these instances the appearance of the vomits showed that they were not proper contents of the stomach but intestinal contents, which had been forced up through the abdominal pressure and had [thus] been voided. The fluid showed an alkaline reaction and also had quite the aspect of intestinal contents." [Trans.]

Some of the fairly numerous subsequent observers who found cholera vibrios in the vomits of the patients particularly Schoebl (1915) considered this occurrence of the organisms to be quite frequent. Indeed as far as the present writer is entitled to judge from his own experiences, it would seem that the rarity of positive results recorded in this respect is more

apparent than real depending largely upon the usually but casual attention given to a bacteriological examination of the vomits It is in accord with this assumption that while Simmonds (1892b) stated that he had found cholera vibrios in the stomach contents at only few of his autopsies Tizzoni & Cattani (1888) recorded such findings in 3 out of 5 cholera victims and Brüloff (1910) in 78% as against over 90% positive results obtained with the contents of the small intestines and 62% with the rectal contents.

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In a study on the invasion of the body by *V cholerae* to which further reference will be made below Sewastianoff (1910) stated that he had found this organism in the gall bladder of 10 out of a total of 18 cholera victims examined by him in 1907 and 1908

Kulescha, reporting—as mentioned above—in 1910 upon a total of 430 cholera autopsies, recorded that he had isolated *V cholerae* from the gall-bladder in 87 out of 159 of these victims, i e in 54 7% The number of positives included 40 out of the 42 instances in which signs of chole cystitis had been manifest at autopsy

Further observations on this point were made by (1) Tanda (1911) who isolated *V cholerae* from the gall-bladder in 2 out of 4 victims (2) Defressine & Cazeneuve (1912) who obtained identical results in all 3 instances examined by them, including one victim showing signs of a catarrhal cholecystitis and (3) Flu (1913) who with the aid of peptone water enrichment, was able to isolate the causative organisms from the gall bladder of 8 out of a total of 18 cholera victims and noted that the viru lence of these cultures practically equalled that of the growths obtained from the intestines.

Greig (1912, 1913a) examining 271 cholera victims, was able to isolate *V cholerae* from the bile of 81 of these individuals, 12 of whom showed distinct pathological changes in their gall-bladder The bile was found to be sterile in 163 out of the 190 cholera negative cases, while in the opinion of Greig the presence of organisms other than *V cholerae* in the remaining 27 instances was partly accounted for by a postmortal invasion He main tained, therefore, that

"The bile, being generally sterile, is, for this reason a very suitable habitat for the delicate comma bacillus, as there are no other organisms to interfere with the growth, such as it would meet in the intestine "

In a further exhaustive study Greig (1914b) recorded that in a number of the bile positive cholera victims he had succeeded also in demonstrating the presence of *V cholerae* (*a*) by bacteriological and histological examina tions in the gall-bladder wall and (*b*) in some of these instances with the aid of histological methods also in the wall of the cystic, hepatic, and common bile ducts Moreover he showed in 9 of these cases with the aid of cultural tests and examinations of histological sections the presence of cholera vibrios in parts of the liver adjacent to pathologically altered intrahepatic bile passages.

Coulter (1915) reporting on the examination of numerous cholera victims stated that on two of these occasions he had found in the gall-bladder wall organisms which though often showing evidence of involu tion appeared to be morphologically identical with *V cholerae* That these organisms were of a true nature was rendered practically certain by the results obtained by Schoebl (1915) when examining 39 gall-bladders removed from the dead bodies of cholera victims by Coulter after the

common bile duct had been ligatured it was possible to cultivate cholera vibrios from 17 of these 39 specimens only 3 of which showed gross altera tions (see page 479 above)

A question of considerable importance is whether instances do occur in which cholera vibrios while not demonstrable in the intestinal contents may be cultivated from the gall bladder of the victims The data available in this respect may be tabulated thus

	Total number	*Cholera vibrios found in*					
		Intestine & gall-bladder		*Intestine only*		*gall-bladder only*	
		number	*( )*	*number*	*( )*	*number*	*( )*
Kulescha (1909)							
Cholera victims	80	27	33 7	31	38 7	22	27.5
Crowell & Johnston (1917)							
Cholera victims	209	125	59 8	72	34 4	12	5 7
Cholera carriers (detected at autopsy)	32	10	31.2	8	25.0	14	43 7

In 4 out of 5 further dead bodies of individuals known to have been cholera carriers during life, negative bacteriological results were obtained at autopsy In the fifth instance *V cholerae* could be isolated from the bile while vibrios not agglutinable with cholera immune serum were cultivated from the small intestine.

These findings make it clear that, when examining the dead bodies of cholera suspect individuals cultivations should be made from the gall bladder as well as from the intestinal contents

While the comparatively quite frequent presence of *V cholerae* in the gall bladder of the victims is generally admitted the problem as to when and how the organisms invade this organ has been the subject of consider able debate.

With regard to the former question it was held by some of the early observers that the presence of *V cholerae* in the gall bladder as well as any other extra intestinal appearance of this organism was the result of an invasion taking place after death or during the often prolonged agonal period of the disease. This view which was expressed in a general manner by Diatroptoff (1894) seems also to have been adopted as regards the gall bladder by Lesage & Macaigne (1893) who—as noted above—were able to demonstrate the presence of cholera vibrios in the bile of some victims dissected after 4 hours but failed to do so in case of the dead bodies autop- sied immediately after death

However while one must admit the possibility that *V cholerae* may enter the gall bladder during the agonal period or after the death of the victims, there can be not the least doubt that such an invasion is also apt to take place at an earlier stage of the disease Irrefutable proof of the latter contention is furnished by the observations recorded above, which have shown that the presence of cholera vibrios in the bile could be associated with marked reactions on the part of the gall bladder and with a penetration of the organisms into the walls of this organ Further as stressed by several

workers, e g., by Defressine & Cazeneuve (1913) and by Schoebl (1915) observations on cholera convalescents and cholera carriers in general lead to an analogous conclusion Schoebl convincingly stated in this connexion

" Considering the lengthy period of infectiveness found in certain instances of cholera carriers and the periodic reoccurrence of cholera vibrios in the stools of convalescents, theoretically it would be difficult to believe that the cholera vibrio would live for such a length of time free in the intestinal tract where the competition with the normal inhabitants and other factors render the conditions unfavourable to its vitality

" The tidal occurrence of the cholera vibrios in the stools of convalescents who become carriers, seems to indicate a focus communicating with the alimentary canal, where the vibrios multiply and are being discharged into the digestive tract. At times and under certain conditions they appear in the excreted faeces in numbers large enough to be detected by the usual methods."

A unique observation by Valk (1915) definitely demonstrated that cholera vibrios are capable of invading the gall-bladder of non moribund patients the sufferer in question, whose stools had become free from *V cholerae* had to be operated on account of a cholecystitis Cholera vibrios could be isolated in pure culture from the bile and continued to be excreted with it through the fistula made for a period of at least four weeks after the operation

In answer to the question how the cholera vibrios penetrated into the gall bladder some workers postulated that in addition to or even instead of entering directly from the intestine, their invasion was effected by other routes Thus Kulescha (1910), pointing out that he had been able to isolate cholera vibrios from necrotic foci in the liver apparently unconnected with the biliary passages, maintained that an invasion of the gall bladder by the blood stream also played an important role. Similarly Sewastianoff (1910) expressed the opinion that *V cholerae* could enter the gall bladder both by the " ascending " route from the intestines and by the " descending " route through the blood stream He thought that the former was the case in instances where the cholera vibrios were found in the bile in association with other organisms, whereas the presence of *V cholerae* in pure culture indicated a haematogenous infection of the gall-bladder Greig (1913a) giving initial attention to the problem presently under review stated

" In typhoid fever the b typhosus, after circulating in the blood stream for a time, deposits locally in various tissues and appears to gain access to the bile from the blood stream. In cholera it may be that the entrance is from the intestine, cholera vibrios being very abundant in the (rice water) contents of the small intestine, differing in this respect from the b. typhosus the observations of Brûloff, however who found the cholera vibrio in the blood, and, also, my own observation, that the comma bacillus may occur in the lung, dealt with later in this paper have to be remembered, it may hereafter be shown that the cholera vibrio like the b. typhosus gains an entrance to the bile by the blood stream."

Reporting afterwards on the occurrence of *V cholerae* in the mesenteric lymph-nodes, Greig (1914a, 1914b) felt entitled to postulate that the

organisms might reach the liver and the bile through the lymph channels However, Nichols (1916) pointing out with full reason that it was difficult to see how an entrance of the infection by this route might be effected once more stressed the importance of an invasion of the gall bladder through the blood-stream

A determined stand against the latter view was taken by Schoebl (1915) and by Crowell & Johnston (1917) Schoebl discussing the comparative importance of a direct and a haematogenous infection of the gall bladder, stated

"The facts that the bile passages show marked pathological changes while the liver tissue proper exhibits as a rule only signs of toxic effect the high percentage of infected gall bladders, and the rarely encountered evidence of a bacteraemic stage of cholera infection speak in favour of the first mentioned [i.e., the direct intestinal] mode of infection "

Schoebl stressed in support of this contention that, as proved by the repeated demonstration of cholera vibrios in the stomach contents and their presence even in the vomits the organisms were quite capable of penetrating into the proximate portions of the digestive tract.

Crowell & Johnston considering the divergent views held by previous observers, came to the conclusion that

"While the evidence is conflicting, it must be admitted that occasionally the disease may be a septicaemia, but that under ordinary conditions the gall bladder is more probably infected through the bile ducts from the duodenum "

The validity of this contention was confirmed by the personal experiences of Crowell & Johnston, who failed to isolate the causative organisms from any part of the body of the numerous cholera victims examined by them outside the intestines the biliary passages, and the gall bladder

No further evidence has become available to invalidate the correctness of the views held by Schoebl and the two last mentioned workers On the contrary Snijders (1922) detecting *V cholerae* in the bile of 4 out of 5 cholera victims, but not in their blood reasserted that at least as a rule the invasion of the gall bladder took place directly from the intestinal tract and not via the blood-stream.

Pancreas

An invasion of the pancreas by *V cholerae* seems to have been recorded solely by Girode (1892) and by Greig (1914a) Girode found in the case of a victim who as mentioned above showed at autopsy also an atrophic cirrhosis of the liver the pancreas increased in size firm and somewhat knobby The protuberances in question showed spots of marked congestion with yellowish centres which latter were found to correspond to small pancreatic ducts filled with a thick fluid rich in cellular elements including leucocytes Cholera *vibrios could* be cultivated from this as well as from

the bile and also from the liver substance Girode entertained no doubt that the invasion of the pancreas as well as that of the gall bladder was due to a direct extension of the infection from the intestinal lumen.

Greig (1914a) demonstrated in 2 out of 3 instances the presence of *V cholerae* in the pancreas of cholera victims, apparently without finding gross changes in the organ.

Mesenteric lymph-nodes

Sewastianoff (1910) recording positive results in 6 out of 8 cholera victims examined in respect of the mesenteric lymph nodes, seems to have been the first worker to demonstrate the presence of *V cholerae* in these nodes This observation was confirmed in a few instances by Greig (1914a) and later by Chatterjee (1939a) who stated that he had established the *presence of the organisms in the mesenteric lymph nodes in not less than* 25 % of his 85 cholera autopsies as against 70% positive cultural results from the intestinal contents and 60% of successful cultivations from the bile.

It would appear therefore, that an invasion of the mesenteric lymph nodes by *V cholerae* is not infrequent. However even though Sewastianoff claimed to have isolated the organisms from the thoracic duct in 2 instances, it is generally held that the lymphatic system does not usually serve as a channel for the general distribution of *V cholerae*

Other organs

Dealing in his 1884 report with the etiology of cholera Koch categorically refuted the idea that this disease was the result of a generalized infection. For he declared, it was

"a peculiar phenomenon, that the comma bacilli remain restricted to the intestine. They do not pass into the blood, not even into the mesenteric lymph-nodes." [Trans.]

While, as has been discussed above, an invasion of *V cholerae* into these lymph nodes was demonstrated comparatively late, evidence of a penetration of the organisms into other parts of the body outside the alimentary tract was adduced soon after Koch had made the statement mentioned above. The early findings made in this respect were diligently summarized by Sewastianoff (1910), whose tabulation it seems well to reproduce in a modified form

Author	Findings
Doyen (1884)	Claimed (*a*) to have obtained positive results when cultivating in gelatin small pieces of the liver spleen, and kidney of 3 cholera victims, *V cholerae* growing as well as contaminating organisms and (*b*) to have found also in several instances organisms morphologically identical with *V cholerae* in histological sections of the liver and kidney
Finkler & Prior (1885)	Demonstrated according to Sewastianoff the presence of cholera vibrios in the liver and the blood of cholera victims.

Author	Findings
Raptschewsky (1885)	Isolated according to Tizzoni & Cattani (1888) cholera vibrios in pure culture from the liver of some victims.
Tizzoni & Cattani (1888)	Stated (a) that they had isolated in 2 instances cholera vibrios from the subarachnoid fluid of victims and (b) that they had obtained cultures of *V cholerae from the heart blood and serous cavities* as well as from the intestinal contents of a 5-month-old foetus of a cholera-affected patient In the case of 2 cholera victims the causative organisms seemed to be present in blood smears, but cultivations gave a negative result.
Rekowsky (1892)	As summarized by Sewastianoff Rekowsky examined 14 cholera victims, obtaining positive bacteriological results, not only from the bile but also 7 times from the liver 6 times from the heart, 4 times from the blood 3 times from the spleen, 7 times from the kidney 3 times from the brain and the spinal cord 4 times from the subarachnoid fluid, and one time from a muscle.
Rommelaere (1892)	Demonstrated, as stated by Sewastianoff in some instances cholera vibrios in the liver blood, lung, and kidney
Fischer (1893)	Claimed to have isolated in one instance cholera vibrios not only from the intestine, but also from pieces of the liver lung, and spleen, which had been washed in 1 per 1000 mercury bichloride and had been opened under aseptic precautions. Histological examination showed small numbers of cholera vibrios in liver and spleen sections.
Lesage & Macaigne (1893)	Observed a "cadaveric" invasion by *V cholerae* in 6 out of 18 cholera victims, who had died in the algid stage of the disease and had been autopsied more than 4 hours after death—the organisms being present "in all organs" 3 times and only in the bile in the other 3 dead bodies. No positive results were obtained (a) from the liver blood, and spleen or from the bile of 14 cholera victims who having succumbed in the algid stage, had been dissected immediately after death (b) in the case of 13 victims who had died in the same stage and were autopsied after 2-4 hours and (c) from the dead bodies of 3 individuals who were dissected immediately after they had died in the stage of reaction.
Bordoni-Uffreduzzi & Abba (1894)	Recorded that they had isolated an atypical strain of *V cholerae* from the heart blood and the spleen as well as from the intestinal contents of a cholera victim succumbed 48 hours after onset of the disease and dissected 5 hours later
Diatroptoff (1894)	Found, when making cultures from the organs of 5 cholera victims, the causative organisms 5 times in the lung and liver and 3 times in the kidney and one time out of 4 instances also in the blood.

Note Babes (1885) recorded that he had obtained a culture of *V cholerae* from the kidney of one out of 5 cholera victims, but considered this positive result to have been due to an accidental contamination from the intestinal contents.

The observations made regarding the problem presently under review by subsequent workers may be summarized thus

Further to the findings of Tizzoni & Cattani in a foetus described above Lwschitz (1909) recorded the isolation of *V cholerae* from the

the bile and also from the liver substance Girode entertained no doubt that the invasion of the pancreas as well as that of the gall bladder was due to a direct extension of the infection from the intestinal lumen.

Greig (1914a) demonstrated in 2 out of 3 instances the presence of *V cholerae* in the pancreas of cholera victims, apparently without finding gross changes in the organ.

Mesenteric lymph nodes

Sewastianoff (1910) recording positive results in 6 out of 8 cholera victims examined in respect of the mesenteric lymph nodes, seems to have been the first worker to demonstrate the presence of *V cholerae* in these nodes This observation was confirmed in a few instances by Greig (1914a) and later by Chatterjee (1939a) who stated that he had established the presence of the organisms in the mesenteric lymph nodes in not less than 25% of his 85 cholera autopsies as against 70% positive cultural results from the intestinal contents and 60% of successful cultivations from the bile.

It would appear therefore, that an invasion of the mesenteric lymph nodes by *V cholerae* is not infrequent However even though Sewastianoff claimed to have isolated the organisms from the thoracic duct in 2 instances, it is generally held that the lymphatic system does not usually serve as a channel for the general distribution of *V cholerae*

Other organs

Dealing in his 1884 report with the etiology of cholera, Koch categori cally refuted the idea that this disease was the result of a generalized infection For he declared, it was

"a peculiar phenomenon, that the comma bacilli remain restricted to the intestine. They do not pass into the blood, not even into the mesenteric lymph-nodes." [Trans.]

While, as has been discussed above, an invasion of *V cholerae* into these lymph nodes was demonstrated comparatively late, evidence of a penetration of the organisms into other parts of the body outside the alimentary tract was adduced soon after Koch had made the statement mentioned above. The early findings made in this respect were diligently summarized by Sewastianoff (1910) whose tabulation it seems well to reproduce in a modified form

Author	Findings
Doyen (1884)	Claimed (*a*) to have obtained positive results when cultivating in gelatin small pieces of the liver spleen, and kidney of 3 cholera victims, *V cholerae* growing as well as contaminating organisms and (*b*) to have found also in several instances organisms morphologically identical with *V cholerae* in histological sections of the liver and kidney
Finkler & Prior (1885)	Demonstrated according to Sewastianoff the presence of cholera vibrios in the liver and the blood of cholera victims.

were found by him in the various organs of 14 cholera victims dissected at intervals varying from 0-26 hours after death was as follows

Organ	*Number of positive findings*	*Number of pure cholera cultures*	*Organ*	*Number of positive findings*	*Number of pure cholera cultures*
Gall-bladder	10	7	Oesophagus	2	1
Heart blood	8	3	Ductus thoracicus	2	—
Mesenteric lymph-nodes	6	3	Serous cavity (? Peritoneum)	1	—
Kidney	5	3	Lung	1	—
Spleen	4	1	Cerebrospinal fluid	1	—
Liver	3	1	Urine	1	?

Sewastianoff pointed out that these figures were not comparable because when dealing with the different dead bodies he had made cultures from different sets of organs Nevertheless, he felt convinced that the cholera vibrio was, on the whole, most often met with in the gall bladder, the mesenteric lymph-nodes, and the blood

It has to be noted that in the case of the single victim dissected immediately after death the cholera vibrios were found " in all organs " except the urinary bladder and apart from the lungs were met with in pure culture As shown by the above table other instances in which *V cholerae* grew in pure culture were less frequent. However while admitting that the occurrence of impure growths increased *pari passu* with the delay in performing the autopsies, Sewastianoff maintained like Brülloff that

" the difference in time between death and autopsy does not influence the frequency with which cholera bacilli can be demonstrated in the internal organs." [Trans.]

In view of the contention of Sewastianoff that the invasion of the internal organs took place *intra vitam* it is curious to note that as a rule the organs found to be free from *V cholerae* yielded growths of other organisms.

Tanda (1911) examining the dead bodies of 4 cholera victims with the aid of cultivations from the heart blood liver and kidney as well as from bile obtained growths of *V cholerae* once from the bile and the kidney and in a second instance from the heart blood the liver the spleen and the kidney

In the first of several articles dealing with the problems presently under review Greig (1912, 1913a) recorded that he had demonstrated the presence of organisms morphologically identical with *V cholerae* in smears and sections from the pneumonic patches met with at autopsy of a cholera victim He soon confirmed (see Greig 1913b) this observation by the isolation of cholera vibrios from the lungs as well as from the stools and bile of another victim Organisms morphologically resembling *V cholerae* could also be demonstrated in sections from the kidney of this dead body and Greig also shortly referred to another instance in which cholera vibrios had been cultivated from the kidneys.

intestinal contents of the stillborn child of a cholera patient in the 8th month of pregnancy the *material in question having been obtained from a loop of* the small intestine which had been removed by laparatomy Liwschitz s *short note furnishes no clue as to in what way the bacteriological diagnosis* was confirmed but apparently the examination was made in a careful *manner*

That, however the observations made by Tizzoni & Cattani and by Liwschitz were exceptional *is proved by the already recorded experiences* of Simmonds (1892b—see page 487) and by further findings of Schoebl (1915) who was unable to isolate *V cholerae* from the organs of 4 stillborn children and of one foetus of cholera affected women. He obtained in one of these instances, in which the foetal sac had been broken prematurely a positive result from the amniotic fluid

As already alluded to Michailow (1909) stated that he had found in sections from the brain and the spinal cord respectively of two cholera victims, autopsied soon after death, agglomerations of organisms showing the typical morphological features of *V cholerae* No cultures from the central nervous system had been made in these two instances and cultivations from the spinal cord and the cerebrospinal fluid resorted to in the case of 2 other victims dissected after 6 hours and 25 hours respectively gave a negative result. Nevertheless, Michailow felt entitled to emphasize the importance of a generalization of cholera infection and particularly that of a penetration of the causative organisms into the central nervous system, which latter was in his opinion responsible for the production of the cholera syndrome Michailow s chief Bechterew (1910) while admitting the validity of the former s histological findings, warned against the acceptance of this far-fetched postulation As has been noted already Michailow himself admitted in his 1913 study that the results of his bacteriological observations were not conclusive

Brüloff (1910) bacteriologically examining the autopsy material of *Kulescha (1909 1910) reached the conclusion that in the dead bodies of* cholera victims the causative organisms were met with not merely *in the intestinal tract, but also in the blood and the other organs.* Their incidence in the latter was 16 ‰, that in the blood and the urinary bladder about 40%. In Brüloff's opinion the frequency with which *V cholerae* was found in the dead bodies outside the intestinal tract was not influenced by differences in the length of time at which the autopsies were made after death however the purity of the growths of *V cholerae* was adversely affected by prolongations of the interval between death and dissection. Brüloff refuted, therefore, the idea that the penetration of the organisms beyond the gastro-intestinal tract was merely a postmortal phenomenon However she did not wish to suggest that cholera was a truly septic infection

In an article already quoted several times in the present chapter Sewastianoff (1910) stated that the frequency with which the causative organisms

the presence of *V cholerae* in the organs outside the gastro-intestinal tract and its annexes was due to invasions taking place before death or before the agonal period

In regard to the first of these questions it has to be stated that most of the workers enumerated above appear to have been quite aware of the danger of an accidental contamination of the materials they collected for cultivation from the organs outside the gastro-intestinal system and consequently took precautions to prevent it. Hence while it is impossible to deny that such a contamination through the intestinal contents may have been responsible occasionally for false positives it seems legitimate to maintain that generally speaking this source of error did not play an important role, and in the case of certain series of investigations presumably none at all

Since the observations made regarding a distribution of *V cholerae* beyond the gastro-intestinal system go back to the first years after the discovery of this organism, when the only quite reliable method for its identification—namely serological tests—was not yet available or at least not universally used it would seem *a priori* that the value of the early findings supported merely by the outcome of bacterioscopic and cultural tests and of the equally not quite reliable cholera red reaction is questionable Actually however it would be wrong to overrate the importance of this potential source of error For practically all the positive findings recorded above were made at times of established cholera outbreaks and in the case of victims in whom the presence of the disease had been demonstrated through examination of their intestinal contents or their stools. It was thus possible to compare the character and the reactions of the growths obtained from the various organs outside the gastro-intestinal system with the corresponding results arrived at through an examination of the faeces or intestinal contents. Generally speaking, there seems, therefore little reason to question the authenticity of even the early findings made in regard to the problem presently under review

The question however to what extent the general distribution of *V cholerae* was the result merely of a postmortal or agonal invasion of the organs concerned instead of an invasion taking place earlier deserves the greatest attention. While as noted above a few workers admitted or even stressed the occurrence of a postmortal distribution of *V cholerae* several other observers strongly denied the occurrence or at least the frequency of this phenomenon

Though the arguments brought forward by Michailow (1909) and by Sewastianoff (1910)—the principal advocates of the latter view—were mostly rather far fetched one must agree with them and their supporters that the penetration of *V cholerae* beyond the gastro-intestinal system was by no means invariably the result of a postmortal invasion. At the same time, however there can be no doubt that the writers bent upon disproving

In a further publication, Greig (1914a) stated that he had been able to undertake an exhaustive bacteriological examination of the organs of 9 cholera victims autopsied soon after death He thus described the technique used for this purpose

"Portions of the organs were removed with sterilised instruments, and to eliminate any possibility of accidental contamination the pieces of tissue were dipped in alcohol and flamed. They were then placed in peptone water and broken up with a sterile glass rod. The flasks containing the tissues were placed in the incubator at 37° After 6 and 24 hours subcultures were made on Dieudonné blood agar In the case of the bile it was drawn off with aseptic precautions and spread on ordinary agar slopes. Then the gall-bladder was opened and the bile washed away with sterile normal saline, a piece of the wall was cut out and dipped in alcohol and flamed and then placed in peptone water as in the case of other organs. Smears were made from the various organs and portions were fixed in alcohol for section cutting."

Results of culture tests made by Greig in the course of his 9 investigations with the aid of the above described technique may thus be summarized

Organ	*Number of positives*	*Organ*	*Number of positives*
Bile	8	Brain and plexus choroideus	1
Gall-bladder wall	5	Heart wall	5
Pancreas	2	Lung	9
Mesenteric lymph-nodes	5	Kidney	9
Liver	8	Wall of urinary bladder	4
Spleen	7	Urine	3

Note The number of instances in which pure cultures of *V cholerae* were obtained is not given.

Jacobitz (1915) examining the blood of 5 cholera patients and of 7 victims to the disease with the aid of peptone water enrichment for the presence of *V cholerae* obtained positive results only in the case of 2 of the dead bodies. One of these 2 victims had succumbed after an illness of about 5 days and had been dissected 6 hours after death, while the second had died on the day of attack and had been autopsied 16 hours afterwards.

As has been noted above Crowell & Johnston were in the course of their numerous investigations unable to demonstrate the presence of the causative organisms in the dead bodies of cholera victims outside the intestinal tract, the biliary passages, and the gall-bladder

Performing 53 autopsies of cholera victims immediately after death, Cantacuzène (1920) was able to isolate the causative organisms from the heart blood in only 5 instances.

As will be gathered from the findings recorded above a considerable number of workers claimed that a generalization of the infection by *V cholerae* was more or less frequent. In order to assess the validity of these claims, it is necessary to consider (1) how far the various workers took precautions against an accidental contamination of their culture materials by the intestinal contents of the victims (2) to what extent they succeeded in establishing the true cholera nature of their growths and (3) how far

However even if one were ready to accept the idea that the lymphatic system did play a role in the general distribution of the organisms it would be impossible to see how the various organs except the mesenteric lymph nodes, could have been reached by *V cholerae* without a subsequent intervention of the blood stream

Jacobitz (1915) while as noted above, obtaining positive results with the blood of 2 out of 7 cholera victims failed like the above mentioned observers to obtain growths of *V cholerae* when cultivating blood samples collected from 5 cholera patients

Dealing with the problem presently under review, Nichols (1916) postulated that in cholera there existed a portal rather than a general septicaemia and that consequently better results might be obtained if instead of the peripheral blood the blood of a mesenteric vein could be used for cultivation However this assumption is not in accord with the observations of Brüloff (1910) who as quoted by Jacobitz, obtained in the case of cholera victims but slightly better results when making cultures from the portal vein instead of the heart blood (40% isolations of *V cholerae* from the portal blood as against 38% in the case of the heart blood)

Since it might be argued that a vibrionaemia, though apt to occur might be of a passing nature great attention has to be paid to the results arrived at by De Monte & Gupta (1938) when inoculating 5-ml amounts of the blood of 26 cholera patients into 100-ml quantities of peptone water and also making direct cultures on agar For even though the samples in question were collected soon after the onset of the disease (3-9 hours after the sufferers had fallen ill) results of cultivation were always negative

In support of the findings just recorded, observations on the appearance of *V cholerae* in the urine of cholera patients which one would expect to have been frequent were a vibrionaemia of common occurrence, were actually few and far between The evidence available in this respect may be summarized thus

Sewastianoff (1910) reported upon 31 examinations, stating that he usually collected his urine samples with the catheter but that he sometimes utilized the last drops of urine which had been voided by male patients after careful disinfection of the praeputium and glans penis with bichloride of mercury Cultivation of these samples yielded growths of *V cholerae* in the case of 2 male and 3 female patients In one of the former whose history was given in some detail the vibrionuria was found to last 4 days After that time the urine of this sufferer found to be slightly acid and free from albumen, still gave a cholera red reaction for a few days.

As briefly stated by Cano (1913) he and Wiener had been able to demonstrate the presence of *V cholerae* in the mucosa of the urinary bladder of a male cholera victim and had also succeeded in finding cholera vibrios in the scanty urine obtained through catheterization from a female patient

the latter failed to pay due attention to the probability of an *agonal* invasion of the general system by *V cholerae* For it is certain that during the often prolonged algid stage of cholera, which with much reason has been likened to a stage of "living death" conditions are favourable for a bacterial invasion and it is consequently most likely that the penetration of *V cholerae* into the general system is particularly apt to take place during this state of nearly abolished vitality At the same time, however it would be unwarranted to deny that such an invasion can take place also earlier in the disease, the sole point at issue being whether this or an agonal invasion is more common

It has to be noted in the latter connexion that observations reliably proving the existence of a vibrionaemia in cholera patients have been made upon only one occasion [1] Ling (1932) described the case of a patient suffering from typhoid fever, from whose peripheral blood cholera vibrios were isolated on two occasions besides typhoid bacilli and from whose heart blood obtained by puncture after death *V cholerae* was cultivated together with *Ps pyocyanea*

Ling was unable to decide whether the individual in question had suffered from a cholera attack followed by a secondary infection with *S typhosa* or—what seems on the whole more likely—had been a cholera carrier in whom typhoid infection had facilitated an entry of *V cholerae* into the blood-stream

In marked contrast to the findings obtained by Ling under altogether unusual conditions, cultivations made by several other workers with the blood of typically affected cholera patients gave invariably negative results

Sewastianoff (1910) recorded in this connexion that he had noted in the case of 2 out of 30 such blood samples in the initial peptone water cultures and subcultures some turbidity which appeared to be conditioned by the presence of a small number of organisms morphologically resembling cholera vibrios Since, however he was unable to produce growth of these suspect organisms on solid media, he admitted that the examination of his 30 samples, though "almost successful" in two instances, had given negative results—a conclusion with which one must thoroughly agree

Similarly Greig (1914b) admitted that he had invariably obtained negative results when cultivating blood samples collected from numerous cholera patients He argued therefore, that a distribution of the organisms through the lymph channels accounted for the invasion of the general system by *V cholerae* which, in his opinion, was of frequent occurrence

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As noted above, Tizzoni & Cattani (18[illegible]) stated that they had seen in 2 instances organisms showing the morphological features of *V cholerae* in smears from the blood of cholera patients, but were unable to confirm these observations through cultivation of the blood samples in question.

Pasricha and colleagues (1936) recovered through liver puncture *V cholerae* from a patient who in the course of a cholera attack had developed icterus and a marked enlargement of the liver Presumably however the presence of cholera vibrios in this organ was the result of local extension of the infection from the intrahepatic bile ducts instead of being due to a vibrionaemia. It speaks for this assumption that cultures from the blood and the urine of this patient gave negative results.

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In support of the findings just recorded observations on the appear ance of *V cholerae* in the urine of cholera patients, which one would expect to have been frequent were a vibrionaemia of common occurrence were actually few and far between The evidence available in this respect may be summarized thus

Sewastianoff (1910) reported upon 31 examinations stating that he usually collected his urine samples with the catheter but that he some times utilized the last drops of urine which had been voided by male patients after careful disinfection of the praeputium and glans penis with bichloride of mercury Cultivation of these samples yielded growths of *V cholerae* in the case of 2 male and 3 female patients In one of the former whose history was given in some detail the vibrionuria was found to last 4 days After that time the urine of this sufferer found to be slightly acid and free from albumen still gave a cholera red reaction for a few days.

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Positive findings in the urine of cholera patients were also recorded by Greig (1913b) who claimed to have found cholera vibrios in 8 out of 55 of the samples examined. However as justly stated by Chatterjee (1941)

"owing to the absence of complete details in his [i.e., Greig's] procedure, chances of contamination cannot be ruled out."

While this objection seems not justified in the case of Sewastianoff's and Cano's observations, the great rarity of the presence of *V cholerae* in the urine of the patients is well illustrated by (*a*) the entirely negative results obtained by Kulescha (1910) when bacteriologically examining numerous urine samples collected under strictly aseptic conditions from cholera patients (*b*) the observations of Schoebl (1915) who failed to find the causative organisms in 41 urine samples from 27 cholera patients or convalescents and (*c*) further investigations by Chatterjee & Malik (1938) who were unable to demonstrate the presence of *V cholerae* in urine samples collected with the aid of catheterization within periods of 1 7 days after onset of the disease from 122 patients Takano Ohtsubo & Inouye (1926) likewise stated that the workers in Japan had never been able to find cholera vibrios in the urine or in the blood of cholera patients

Taken as a whole, the experiences gained through examinations of the blood and urine of cholera patients lend strong support to the view that a pre agonal generalization of the infection instead of being of common occurrence, is exceptionally rare

Pathogenesis

Though it is impossible to deal exhaustively with the pathogenesis of cholera before the problems of the clinical pathology of the disease have received attention in the eighth chapter of this book the following subjects being germane to the present disquisition may with advantage now be discussed.

Portal of entry of the infection

As has been stated above (see page 407) Sanarelli (1923a) suggested on account of experimental findings that cholera was the result not of an intestinal infection but of an entry of the causative organisms through the faucial organs followed by their distribution through the blood-stream. As was pointed out however for various reasons Sanarelli's assumption was not acceptable. It may be claimed that the validity of this contention is fully supported by the observations now recorded on the pathology of the disease and the distribution of *V cholerae* in man It would be redundant, therefore elaborately to deal with Sanarelli's hypothesis which may be said to be merely of historical if of any interest.

Role of the cholera toxin

Reporting on his findings at the 1884 cholera conference Koch maintained that the signs and the course of the disease in man could be explained by the assumption that the comma bacilli produced a specific poison adding that

"the action of the poison becomes manifest partly in an immediate manner inasmuch as through it the epithelium and in the most severe cases also the upper strata of the intestinal mucosa become necrotized partly it is resorbed and acts on the whole organism, particularly on the circulatory organs which as it were become paralysed. The symptom complex of the cholera attack proper usually considered to be due to dehydration and a concentration of the blood is in my opinion to be considered essentially as an intoxication. For it is not rarely met with also when during life comparatively very small amounts of fluid have been lost through vomiting and diarrhoea and when immediately after death the intestine as well contains but little fluid." [Trans]

Even apart from the fact that as has been discussed in the first section of Chapter 4 Koch erred when assuming that the cholera vibrios produced an exotoxin the validity of his hypothesis has remained the subject of considerable debate It was soon assailed by Emmerich & Tsuboi (1893 see also Emmerich 1893 and 1911) who reached the astonishing conclusion that the cholera syndrome was the result of nitrite poisoning due to the capability of *V cholerae* to reduce *in vivo* as well as *in vitro* the nitrates to nitrites However though winning the support of a few writers quite particularly that of Sticker (1912) the validity of this assumption was categorically refuted by several other workers, such as Klemperer (1893) Pfeiffer (1894) Liebermeister (1896) Choukevich (1911) and Kolle & Prigge (1928) Most important among these objections to the theory of Emmerich & Tsuboi and of Emmerich are the following

Klemperer (1893) pointed out *inter alia* that (*a*) cholera vibrios which had been bereft of their vitality and consequently of their ability to reduce nitrates to nitrites, still produced a typical fatal syndrome in experimental animals and (*b*) the virulence of the organisms could become lowered without impairment of their power to cause a nitrate reduction

Though examining with the aid of Proskauer not only animals which had succumbed to cholera but also those killed at various stages of the infection Pfeiffer (1894) was never able to demonstrate the presence of nitrites in the peritoneal exudate of intraperitoneally infected guinea pigs or in the contents of the small intestines after oral infection according to Koch s method

Choukevich (1911) was unable to confirm that, as claimed by Emmerich, guinea pigs which had ingested cholera vibrios together with nitrates succumbed and showed autopsy signs characteristic of cholera whereas controls given either the organisms or nitrates, remained unharmed For though in Choukevich s experience the combined oral treatment proved usually fatal the post mortem appearances in such animals " had nothing

in common with cholera" Moreover young rabbits which, because solely fed with the milk of their mothers, did not ingest nitrates, were nevertheless susceptible to oral cholera infection Such animals showed no or only negligible quantities of nitrates in their intestinal contents

In the opinion of Kolle & Prigge (1928) there existed on the one hand "no demonstrable analogies between the morbid picture of cholera and nitrite poisoning" while on the other hand pathogenic organisms like *S paratyphi B* and *Sh flexneri*, though equalling *V cholerae* in nitrate reducing properties produced clinical syndromes fundamentally different from that of cholera.

While refuting Sanarelli s thesis, Pfeiffer (1894) fully supported the views of Koch drawing attention to the marked epithelial desquamation met with even in the intestines of cholera victims who had been dissected soon after death, Pfeiffer concluded from these observations that

"at least a very considerable damage of the intestinal epithelium was present during life. In this way in man also the preliminary conditions are created which were found necessary in guinea-pigs for the evolution of cholera intoxication. The resorbed cholera toxins produce as in the guinea-pig a drop in temperature, paralysis of the circulation, general muscular debility and partial muscular convulsions, that is, just the symptoms characteristic of the algid stage." [Trans.]

Moreover Pfeiffer emphasized the specific serological changes met with in cholera convalescents served as a direct proof "that these toxic substances actually come into the blood-stream"

Agreement with the views of Koch, which were reasserted in the 1887 report by Gaffky was expressed also by other workers Thus Klemperer (1894) anticipating Pfeiffer held that the appearance of immune bodies in the blood serum of individuals who had been affected by cholera formed proof positive of a resorption of the cholera toxin. Further as has been mentioned above (see page 483) some early observers believed in a circulation of these toxins, because they held them responsible for the production of the renal lesions characteristic of severe cholera attacks.

Kolle & Schürmann (1912 see also Kolle & Prigge 1928) aptly expressed the views of the German school by stating that

"The cholera process is essentially an infective process of the intestinal epithelium with subsequent intoxication. The vibrios multiplying in the lymph-spaces between the epithelial cells supply this toxin when they succumb. If it has come to an epithelial necrosis and desquamation of the epithelium, the toxic substances present in the intestinal lumen also exert an action." [Trans.]

However like Koch (1884) Kolle & Schürmann pointed to the occurrence of cases of cholera sicca, in which diarrhoea and vomiting were absent and, even though an infection of the intestinal epithelium could be demonstrated, no epithelial desquamation was found at autopsy

The indispensability of an epithelial desquamation for the pathogenesis of typical cholera attacks was denied by Stoerk (1916) who as mentioned

before maintained that the process was initiated by a dilatation of the intestinal vessels followed by the transudation of fluid which frequently led to a detachment of the epithelium from the basal membrane not resulting in desquamation He added that

"The histological appearances justify the assumption that the extent of transudation from the vessels of the intestinal mucosa specially from the subepithelial capillaries, into the intestinal lumen is very considerable One could compare the process for example with an inflammatory lung oedema for evidently one has to do with an abnormal permeability of vessel walls damaged by the toxin. I am of the opinion that there exists a most immediate relation between this form of transudation and the watery consistency of the cholera stools. One must realize in this connexion .. the tremendous extent of the interior surface of the intestine (and the corresponding extent of the subepithelial capillary net primarily concerned in the transudation) conditioned by the configuration of the villi in the small intestine " [Trans]

Reference has been made already (see pages 472 and 490) to the views of Goodpasture (1923) who denying the common occurrence of a pre agonal epithelial desquamation held that in cholera

" the great mass of the vibrios is confined to the intestinal lumen and, if toxic substances are formed there directly or indirectly as a result of their growth they are absorbed early in the disease through an anatomically intact mucosa."

An opposite view was expressed by Banerjee (1939) who considering a marked necrosis of the epithelium as one of the characteristic lesions present in the small intestine of cholera victims, thought it probable that this destruction of the epithelial lining accounted for the great drain of body fluids and salts into the intestinal lumen

De Sarkar & Tribedi (1951) came on account of experimental observations to a view similar to that expressed by Stoerk for they stated that in human cholera sufferers

"The endotoxin, liberated by the death and disintegration of the vibrios, through a local action on the capillaries of the intestinal mucosa, increases their permeability causing an outpouring into the lumen of the gut of plasma sometimes mixed with red blood cells. The toxin is also absorbed and thereafter exerts a specific toxic effect on the submucosal capillaries of the small intestine "

When trying to draw conclusions from the rather discrepant opinions of the observers enumerated above one must incline to the belief that an action of the cholera toxin on the submucous capillaries of the small intestine is of primary pathogenetic importance while the epithelial desquamation plays a secondary and not indispensable role The results of experimental studies like those of Burrows, Wagner & Mather (1944) and of De & Chatterjee (1953) referred to above (see pages 417 and 449) fully support this contention

While notwithstanding the dissensions discussed above it is generally accepted that the intestinal manifestations of cholera are due to an action of the endotoxin of *V cholerae* the question to what extent this endotoxin

is responsible for the production of the general symptoms and signs of the disease has remained the subject of much doubt.

It seems well to consider in this connexion first an hypothesis which though commonly ascribed to Sanarelli (1923b) was advanced already by some earlier writers, with particular clarity by Ciaccia (1914) who in an article already referred to spoke of

> the possibility that the cholera vibrio engenders only a local action with the consecutive severe lesions of the intestinal mucosa, which in its turn would create necessary and sufficient conditions for the transition of toxic, possibly in part bacterial products from the intestinal lumen into the circulation [Trans.]

In Ciaccia's opinion among these toxic substances those produced by the *E. coli* group of bacteria seemed particularly important.

Sanarelli (1923b) concluded from experimental observations already noted above (page 431) that

> "The number of points of contact that one finds between the symptomatology and the anatomic lesions in this kind of algidity obtained experimentally in guinea pigs, and the algid state observed in human cholera permits so it seems to me, to class, from now, cholera algidity among the anaphylactic phenomena. Algidity would only be a brusque access after a preparatory phase of sensitization or incubation, represented by the simple and direct action of the vibrios on the mucous membranes of the intestine and then set going by the joint action as unexpected as it is indispensable, of other microbes, or their proteid. These are, as a rule, *B. coli*, staphylococcus, etc., temporary but nearly always habitual, inhabitants of the lymphatics of the alimentary canal. Following upon the enteric action of the vibrios, these others are roused and become exalted in virulence pouring into the blood their antigen and invading, very often, the different viscera." [1]

Sdrodowski & Brenn (1925) while cautiously supporting the validity of Sanarelli's contentions, admitted that it was possible to produce an algid syndrome by sensitization of experimental animals with *E. coli* in place of cholera vibrios. In their opinion therefore this problem

> "fell into the realm of the pathology of algid states which are met with also in some intestinal diseases not caused through cholera vibrios (cholera nostras, gastroenteritis of children) and the etiology of which stands in a close relation with the coli bacilli." [Trans.]

Views similar to those of Sanarelli were also propounded by Banerjee (1939) and by Chatterjee (1939b 1939c). As quoted in the *Tropical Diseases Bulletin* (1939) Banerjee considered the algid stage of cholera to be due to the conjoined action of the cholera vibrio and extraneous intestinal organisms and also suggested that an absorption of histamine from the intestine might be responsible for the fall in blood pressure seen in cholera patients.

Chatterjee (1939b 1939c) laid even greater stress upon a role played in the pathogenesis of cholera by an absorption of histamine, pointing out that (*a*) *V. cholerae* when grown in synthetic media, brought about a complete transformation of histidine into histamine and (*b*) the lesions met

[1] Translation culled from the review of Sanarelli's article in the *Tropical Diseases Bulletin* 1924

with in cholera victims resembled those produced by histamine Generally speaking he was of the opinion that the cholera syndrome was not the result of a toxaemia but was rather due to the creation of an allergic state

Claims that an absorption of *E coli* autolysates from the intestines of cholera patients might aggravate the symptoms of the disease were made by Ghosh & Mukerjee (1941) because they had observed that (*a*) the injection of sterile filtrates of cholera stools into rabbits led to the appearance of considerable amounts of *E. coli* agglutinins in the sera of these animals, and (*b*) the sera of 25 out of a total of 35 cholera convalescents which agglutinated *V cholerae* also agglutinated the *E. coli* strains isolated from these individuals whereas such coli agglutinins were much more rarely met with in healthy persons or in typhoid patients

The views propounded by Sanarelli and by Sdrodowsky & Brenn were sharply criticized by Kolle & Prigge (1928) who reached the conclusion that the results recorded by these workers

"had added nothing certain to the already known facts, experimental findings, and theories through which the pathogenesis and the evolution of the cholera infection, the clinical picture and especially the characteristic complex of symptoms, the algid stage could be explained in a satisfactory manner The importance of the *Bact coli* for the causation of the algid stage and the interpretation of the latter as an anaphylactic shock have not found a satisfactory experimental confirmation" [Trans.]

Similarly an editorial in the *Calcutta Medical Journal* (1939) which followed the article by Chatterjee (1939b) was not in agreement with the views of this worker or with those of Banerjee (1939) declaring that

"At the present state of our knowledge cholera cannot be regarded as an allergic manifestation as it is difficult to imagine specific hypersensitiveness in epidemic diseases."

The writer of the editorial was likewise of the opinion that the supposed production of histamine in the intestinal tract failed to offer a satisfactory explanation for the pathogenesis of cholera.

Weighing the evidence adduced above one must agree with the views of Kolle & Prigge and of the editorial statement just mentioned. For it is impossible to see how the development of an allergic condition could account for the rapidly evolving cholera syndrome and how in particular an action of *E coli* could be held responsible in view of the fact that early in the disease *V cholerae* is often present in the intestines in nearly pure or even quite pure culture Moreover even if the claims of Ghosh & Mukerjee could be accepted as generally valid, their findings furnish no evidence that the *E. coli* autolysates, supposed to be of importance in cholera by these two workers, exert an action early in the disease

Though it was held by some authorities, e g., by Kolle & Prigge (1928) and the editorial writer just mentioned, that a refutation of the postulates of Sanarelli and of similar dissentient opinions (see also Rainsford 1952) automatically reaffirmed the validity of Koch s tenets actually a critical reappraisal of the latter is urgently required.

is responsible for the production of the general symptoms and signs of the disease has remained the subject of much doubt.

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[1] Translation culled from the review of Sanarelli's article in the *Tropical Diseases Bulletin*, 1924

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As will be gathered from his statement in 1884 quoted above Koch felt entitled on account of his recent discoveries to overrule the formerly held opinion that the general signs of cholera were the result of a dehydration and of a subsequent concentration of the blood and to maintain instead that the cholera syndrome was the result of a toxaemia. However Leyden (1892) approaching this problem with the wide outlook of a great clinician, again affirmed the great importance of the formerly held views stating that he

" was not able to recognize in the symptoms of the initial cholera attack the action of an essential toxin ... Very frequently the sensorium remains free until asphyxia becomes fully developed, anyhow for very long. The heart also shows nothing suggestive of a toxin action, for the pulselessness is the result of dehydration. If however the blood again attracts water or as the result of an intravenous infusion, pulse and heart recover fairly rapidly Equally important is the empirically established fact that cholera is followed by but few secondary diseases [*Nachkrankheiten*] and hardly by any referable to a toxin action." [Trans.]

Liebermeister (1896) in his classical contribution on cholera to Nothnagel's handbook on internal diseases, maintained that, as had already been advocated in 1849 by Niemeyer cholera fell into the category of " local " infectious diseases, inasmuch as the causative organisms exerted a direct action only in the intestinal tract. He was willing to admit that the intense intestinal catarrh as well as the necrosis and desquamation of the intestinal epithelia was mainly the result of an early action of the cholera toxin but deplored the tendency to

" consider all or almost all disturbances outside the intestinal tract simply due to toxic actions and thus to underrate the importance of dehydration and nervous impairment of the heart " [Trans.]

Though, as has been noted above, no complete agreement has been reached most modern observers (see for example Burrows, 1948) are in favour of the views of Leyden and Liebermeister rather than of those of Koch and his school. It has already been stressed in this connexion that the results of recent studies on the kidney lesions in cholera strongly spoke against a direct action of the endotoxin on this organ

At the same time however it would be unwise to assume that the cholera toxin exerts exclusively a local action in the intestinal tract. There can be no doubt that an absorption of the toxin does take place and it is most likely that some of the lesions becoming manifest in the course of cholera outside the gastro-intestinal tract, for instance the degeneration of the striated muscles, observed also in other infectious diseases, are due to its direct action This view is further supported by the experiences with the cholera toxin in experimental animals and in tests with isolated organs Hence, overshadowed though it is by the spectacular manifestations due to dehydration and its sequelae a direct role of the toxin in the causation of the general cholera syndrome should not and cannot be disregarded

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Chapter 7

PRACTICAL LABORATORY DIAGNOSIS

Stool Examination

Collection of specimens

Different procedures have to be used for the collection of stool specimens for cholera laboratory work according to whether faeces of patients or of suspected carriers of *V cholerae* have to be examined Moreover a special technique is required for obtaining the material necessary for such examinations from the intestinal contents of supposed victims of the disease

The method usually resorted to when handling the fluid stools of cholera patients is to put adequate quantities of freshly voided faeces—preferably with the aid of small pasteboard spoons of the type supplied by ice-cream vendors—into suitable containers which are then forwarded as rapidly as possible to the laboratory In place of thick walled, small Erlenmeyer flasks or glass jars ordinarily used for commercial samples and recommended early by Dunbar (1896) other containers particularly the earthenware jars cheaply available in Eastern countries may be utilized In fact, as long as the laboratory is situated near to the wards any leak proof container which is provided with a cover fitting tightly enough to avoid spilling, access of flies, and rapid drying of the samples and which can be sterilized by autoclaving or boiling, is serviceable However it would be more expedient to employ in place of such containers the pasteboard boxes recommended originally for quarantine work (McLaughlin 1916) because these if taken immediately before use from the original packages, can be utilized without preliminary sterilization and, after completion of the examination can be discarded easily as well as safely by incinerating them together with their contents

Some modern workers have recommended relying for the examination of cholera dejecta not upon their natural evacuation but upon their collection with the aid of mechanical contrivances Besides the method of swabbing—which will be dealt with below as it is as a rule used in the search for cholera carriers rather than for the examination of patients in the acute stage of the disease—the insertion of glass tubes or of rubber catheters has been

recommended for stool collection. Thus Reimann et al (1946) recorded that, in order to obtain material for cholera diagnostic tests during an epidemic they utilized for each sufferer two glass tubes which "were inserted in turn about 3 inches into the rectum drawn back and forth gently several times until fluid entered the side aperture and collected in the rounded end" One of these tubes after a smear had been taken from its outside surface was placed into alkaline peptone water the other into the preserving medium of Venkatraman & Ramakrishnan (1941) described later in this chapter *to serve as a reserve in case the examination and* subcultivation of the peptone water specimen gave no clear-cut results

The sterile soft rubber catheters to be used for the collection of the fluid stools of patients in the acute stage of cholera should be first lubricated with sterile liquid petrolatum and then inserted 4-5 cm into the rectum The specimen thus voided is collected into a sterile tube or flask.

Whatever method of collecting the specimens is chosen it is imperative *to avoid their coming in contact with disinfectants or with ordinary water* which besides generally not being sterile may contain cholera like vibrios. The greatest possible attention must be paid to the proper labelling of the samples To use merely the bed number for this purpose is altogether unreliable because, when the results of the examination are reported to the ward the bed in question may already be occupied by another patient. Particular care must be taken in this respect when further samples from a previously examined patient are sent to the laboratory

In order to avoid the difficulties and delays attendant upon the collection of stools from suspected carriers of *V cholerae* many of whom may have to be handled simultaneously in quarantine work, several workers e g. Creel (1911) Craster (1913) and Ahuja et al. (1950 1951) recommended obtaining material for laboratory examination from such persons with the aid of rectal swabs McLaughlin (1916) pointed with great reason to the disadvantages of this method it was difficult to obtain sufficient material or even any at all, if the suspected persons were constipated the material on the swab was apt to be scraped off by the anal sphincter during the process of retraction in the case of individuals afflicted with haemorrhoids, in particular the insertion of the swab was quite painful. It is true that some of these drawbacks can be obviated by dipping the swab before insertion into peptone water or some other sterile fluid, or better still, by encasing it in a clipped rubber catheter or rubber tube through the open inner end of which the swab is pushed after the well-lubricated tube has been inserted 3-4 cm deep into the rectum. After faeces have been collected by moving the swab to and fro the swab is drawn back into the rubber casing and the assembly is withdrawn A still more satisfactory but also more tedious procedure is according to Creel (1911) the initial insertion into the rectum of short thick walled glass tubes with rounded edges, which serve as specula for the subsequent introduction of the swabs Nevertheless

on the whole the method of obtaining faecal specimens with swabs seems not an advantageous one all the less so because, according to a small series of observations recorded in the 1949 report of the Indian Research Fund Association the percentage of positive results obtained through their examination alone was below that obtainable with stool samples

The usual procedure of obtaining stool specimens from suspected cholera carriers is to issue them with wide mouthed bottles or jars of a capacity of about ½ 1 fluid ounce (about 15-30 ml) provided with corks or preferably with metal covers, or best with screw-caps Small wooden tin or pasteboard spoons wrapped in paper may be issued together with these containers to facilitate insertion of the stool specimens into the latter but it is better to affix the spoons to the inside of the covers The persons to be examined must be instructed to put a piece of stools of about hazel nut size into the containers and to close them carefully

A definite though often not realized drawback to this otherwise con venient method is that one can be never quite sure whether the collected samples are actually derived from the individuals named on the labels of the containers. The present writer knows of instances where ships stewards obligingly filled the jars supposed to have been issued to the passengers with their own stools.

An alternative method of stool collection used by Müller (1915) was to issue to each of the numerous soldiers he had to examine a piece of thick packing paper measuring 15 × 15 cm, for deposition of their faeces. Wooden sticks were then used to collect pea sized portions of the stools. A similar procedure was successfully used by the present writer to study the incidence of cholera and cholera like vibrios in the population of Shanghai through collection of numerous samples in the public privies

In the official instructions for the collection and transport of cholera suspect objects for examination compiled by Koch and co-authors and promulgated by the Prussian authorities in 1902, it was prescribed that " if no voluntary voiding of stools can be obtained, the same must be effected through the introduction of glycerin "

Recommendations for inducing the passing of stools artificially for the purpose of laboratory examinations were also made by several subsequent cholera workers While Pottevin (1915) suggested the use for this purpose of either (?glycerol) suppositories or enemas with boiled water or purgatives, most authorities advocated the use of the latter McLaughlin (1916) re commended in this respect that passengers who had to undergo quarantine be given magnesium sulfate routinely before breakfast so as to ensure the collection of stool samples However for persons suffering from diarrhoea as well as for children he used large catheters or rectal tubes with several openings at the inner end to obtain specimens, which, like those from the naturally voided stools were put into pasteboard " cuspidors " or similar containers.

It is important to note that the administration of magnesium sulfate to cholera convalescents whose stools had proved bacteriologically negative on two or three previous examinations was found by some workers, such as Tanda (1911) Zirolia (1911) and Piras (1913) to lead more or less frequently to a reappearance of *V cholerae* in the faecal specimens Gohar & Makkawi (1948a) were unable to obtain such results when administering magnesium sulfate to a group of cholera convalescents examined about one month after an epidemic in the village in question had terminated. They added that

"In a few cases duodenal intubation and administration of magnesium sulphate were carried out but this did not alter the result. Apart from the trouble this procedure entailed, it was found that the concentrated magnesium sulphate solution when added to the peptone water medium caused inhibition of the cholera vibrio in a dilution of 1.5 per cent., though it was not bactericidal in as much as 25 per cent. concentration"

However in contrast to these findings, the actual experiences of the earlier workers did not indicate an unfavourable influence of magnesium sulfate on the reappearance of *V cholerae* in the stools. Their method therefore perhaps deserves more attention than it has recently received.

The routine administration of laxatives in quarantine practice as used by McLaughlin is in the opinion of the present writer not recommendable If such mass examinations have to be undertaken there is, with but few exceptions, no difficulty in obtaining stool specimens. If constipated individuals are met with faecal specimens can be quite easily obtained from them with the aid of glycerol suppositories or small enemas

Amending the rather elaborate original recommendations of Koch and co-authors (1902) for the collection of material for bacteriological examination from cholera suspect dead bodies instructions promulgated by the German authorities in 1916 and reprinted by Kolle & Prigge (1928) prescribed the removal of a loop about 10-cm long of the lowest part of the small intestine after double ligature This piece of intestine was to be enclosed in a thick walled, wide mouthed glass jar provided with a ground-in glass stopper or with a well fitting and freshly boiled cork. The jars had to be safely packed before transport to the laboratory

Adequate though this method is, modern workers will prefer to remove material from the small intestine for dispatch to the laboratory at autopsy under aseptic conditions this is best done with a sterile pipette provided with a rubber bulb For the reasons discussed in the preceding chapter it is essential, when dissecting the dead bodies of cholera suspect individuals, also to obtain material from the gall bladder for bacteriological examination.

Preservation of specimens

Discussing the chemical affinities of *V cholerae* Nicholls (1917) recommended preserving cholera-suspect stools by the addition of an adequate amount of sodium carbonate

Panganiban & Schoebl (1918) studied possibilities of preserving cholera suspect faecal specimens through experiments conducted at room temperature (32 C) with artificially contaminated stools They recorded the following results

(a) Glycerol exerted no preserving action the cholera vibrios surviving in 20% or 25% concentrations of this carbohydrate for 4 days only while at a concentration of 30% the organisms were no longer cultivable on the fourth day

(b) Sodium chloride in concentrations of from 0.5% to 5% preserved the cholera faeces throughout the observation period of 5 weeks but in concentrations of more than 5% the organisms were no longer demonstrable after 4-5 days.

(c) Ox bile used in a concentration of 50%, 75% or pure gave identically good results and proved more apt than sodium chloride at a 1% concentration to maintain the viability of cholera vibrios added to the faecal emulsions in small numbers

Notwithstanding these experiences, the method of using sodium chloride solutions for the preservation of cholera-suspect stools was again recommended by Tomb & Maitra (1926) and by Brahmachari (1927) who advised the addition of 4-5 g of the faeces to 30 ml of 1% saline in test tubes 6 inches by 1 inch

The problem of the admissible delay in the examination of stools for *V cholerae* was again studied by Soda et al (1936) who were able to experiment with the faeces of three cholera patients keeping the specimens respectively at 37° C, at an unspecified room temperature and in the refrigerator As was to be expected survival of the organisms was shortest at incubator temperature (37° C)—sometimes not longer than 3 hours—and as long as 8 days in the refrigerator Survival of the cholera vibrios was also found to be longer in diarrhoeic than in solid stools On account of these experiences and of further tests made with artificially contaminated faecal specimens Soda et al recommended that in actual work with the faeces of suspected cholera carriers not less than 5-g quantities of stools should be used for examination and that these should be collected not more than 24 hours beforehand and kept at a low temperature If in sea borne traffic, a ship's doctor was available he should immediately make peptone water cultures with the travellers faeces collected within 24 hours before arrival Otherwise it was recommended that the faecal specimens be preserved at a low temperature after addition of 10 ml of peptone water with a pH of 8 4

Seal (1939) making a comparative study of enrichment methods to which further reference will be made below preserved his stool specimens by adding them in quantities of 2 teaspoonfuls (= 8 10 g) to 20 ml of 2% NaCl solution filled into 100-ml wide-mouthed and glass-stoppered

sterile bottles, afterwards adding 2 3 drops of a N/1 NaOH solution. In the latter part of his study however he increased the quantity of the NaCl solution to 40 ml with a corresponding increase in the inoculum.

A similar technique was used by Read & Pandit (1941) when studying the incidence of cholera and El Tor vibrios in rural areas of India.

Sufficient amounts of stool samples, quite freshly collected in earthenware pots with lids, were put with the aid of bamboo sticks into 50-ml amounts of a 2 % solution of common sea-salt, contained in wide-mouthed, glass-stoppered bottles, to obtain thick emulsions. As a rule, 3-4 drops of a normal NaOH solution were added to the sea-salt solution before inoculation, so as to counteract the usually acid reaction of the stools.

Venkatraman & Ramakrishnan (1941) found that (*a*) a sea salt concentration of 2 % and a pH of 9 2 were most suitable for a prolonged survival of *V cholerae* in the presence of other bacteria and (*b*) cholera vibrios survived in boric acid solution at a concentration of up to 1.5%, whereas *E. coli* and *Aerobacter aerogenes* were completely inhibited by a concentration of 0 6% and, to a considerable extent, even of 0.3%. Accordingly these two workers devised a preserving fluid for the transmission of cholera suspect specimens prepared as follows

" 12.405 g. boric acid (H_3BO_3) and 14.912 g. potassium chloride (KCl) are dissolved in about 800 c.c. of hot distilled water the solution cooled and made up to 1 litre. From this stock solution 250 c.c. are taken, mixed with 133 5 c.c. of M/5 NaOH and the whole made up to a litre. Twenty grammes dried sea-salt (common salt from the bazaar serves equally well) are dissolved and the buffered saline filtered through paper dispensed in 10-c.c. quantities in 1-oz. [28 ml] screw-capped bottles and sterilized in the autoclave."

The two workers added that

" The sterilized buffer has a pH of 9.2 and is found to maintain the same pH for months. The collecting outfit includes, for convenience, a small aluminium spoon which will hold about 1 g. to 3 g. of stool depending on the consistency In use, a spoonful of the stool specimen is well mixed in the buffer which is then mailed."

Reporting on the use of their preserving fluid, Venkatraman & Ramakrishnan stated that in the case of artificially contaminated faeces only a slight initial multiplication of the cholera vibrios took place but that the organisms remained viable in the buffer solution for as long as 62 days. From stool samples collected from a cholera patient on the first day of illness and kept in the preserving fluid, *V cholerae* could be isolated up to the 92nd day i e until the specimens were exhausted. Referring to a field trial of their preserving fluid Venkatraman & Ramakrishnan recorded that during an epidemic

" Two sets of specimens of stools were collected in the preservative from clinically typical cases of cholera. One set was examined immediately on the spot while the other was mailed to the laboratories at Tanjore, taking 4 to 7 days in transit, and occasionally longer Both in the field and in the laboratory platings were made after preliminary enrichment in mannose bismuth-sulphite medium. Sixty-six specimens, including a specimen of vomit, were taken from 60 cases. *V cholerae* was isolated from 64 specimens in the field, and from 60 in the laboratory No case was missed. In two instances,

V cholerae was isolated from mailed specimens, while the immediate examination of specimens taken in the field proved negative. We had the impression that failure to isolate *V cholerae* occurred in those instances where an excess of stool had been added to the preservative causing a drop in the pH"

Venkatraman & Ramakrishnan also obtained apparently satisfactory results when using their preserving fluid for the examination of stools of cholera contacts

The use of this method in the case of suspect stools which had to be examined after some delay was recommended in instructions for cholera laboratory work published by the Public Health Laboratory Service under the auspices of the British Medical Research Council in 1947 It is also noteworthy that Felsenfeld et al. (1951) in the course of a laboratory study to which further reference will be made below, found the average survival time of *V cholerae* in the preserving fluid at present under review to be satisfactorily long, regardless of whether tests were made with pure cultures or with mixtures containing both cholera vibrios and other organisms, including *E. coli A aerogenes* and *Proteus vulgaris*

Pooling methods

As far as can be ascertained Müller (1915) was the first worker to report on the successful use of pooling methods for the detection of *V cholerae* in the stools of large groups of individuals, in his case of battalions of soldiers among whom cholera cases had occurred and who therefore had to be quarantined In order to examine them in a simplified manner he successively emulsified pea-sized pieces of the stools of 10 individuals in 50 ml or sometimes even in only 30 ml of peptone water and then continued with examination of these initial cultures in the usual way Whenever positive results had been obtained by this method the groups in question were isolated and treated with animal charcoal and tincture of iodine the individuals actually found to harbour cholera vibrios were discharged only after further examination of their stools had proved negative three times.

The pooling method was again used by Shahin (1933) for the mass examination of Mecca pilgrims. However though he once succeeded in finding *V cholerae* in a pool of ten stools and isolated on three further occasions cholera like vibrios from such pooled specimens, he was unable to ascertain who among the members of these groups were responsible for the positive results. It is possible that he omitted the now universally adopted procedure of keeping the individual stools which remain after specimens have been taken for pooling until the examination of the pooled material had been completed To rely instead upon repeated collection and examination of individual specimens is rather unsatisfactory in view of the often markedly intermittent character of vibrio excretion by cholera carriers.

The method of examining pooled stool specimens for the presence of *V cholerae* has also been utilized more recently by Gohar & Makkawi

(1948a) and in a large-scale investigation in Madras State in India carried out under the direction of Venkatraman (1949) The first two observers, working in an Egyptian village where an epidemic had ended about a month previously were unable to detect carriers with the aid of pooling tests in a group of people who had not been in direct contact with patients A search for subclinical cholera cases undertaken with the same technique by Venkatraman and his staff also gave entirely negative results, even though Venkatraman & Ramakrishnan s preserving fluid was used in 5-ml quantities for the collection of individual stool samples and although highly specific media, which will be described below were utilized for enrichment and cultivation of the pooled specimens That, however these negative results were due merely to an absence of cholera in the investigated villages and not to any inadequacy of the pooling technique was proved by the examination of 2240 stool specimens from 245 staff members of a hospital where cholera patients were accommodated at the time Cholera vibrios could be detected in the pooled stool specimens of these 245 persons upon five occasions and in all but one of these instances it was possible to identify the carriers in question by referring back to the remnants of emulsified stools left in the collecting bottles. Continued daily examination of their stools, on the other hand, failed to give positive results

These findings as well as the results originally recorded by Müller support the contention that it is legitimate to resort to pooling tests when trying to detect cholera carriers in the course of mass examinations

Macroscopic inspection of stools

General agreement exists that, though as a rule severely attacked cholera patients void rice watery stools during the acute stage of the disease this feature is of no diagnostic importance because it is also quite often met in gastro-intestinal affections due to other causes Moreover it is frequently possible to isolate *V cholerae* from quite uncharacteristic stools not only of carriers but also (*a*) of patients suffering merely from choleraic diarrhoea (*b*) of individuals who though afterwards showing symptoms and signs of a severe cholera attack, are first seen quite early in the disease and (*c*) of sufferers past the acute stage of cholera or convalescing from it. The marked variance in the aspect of bacteriologically positive stools met with in laboratories to which faecal specimens of contacts as well as of cholera patients are sent is well illustrated by a series of observations recorded by Dunbar (1896) who succeeded in isolating *V cholerae* from faecal specimens showing the following macroscopic appearances

Typically rice-watery	10	Watery and bile-coloured	14
Like gruel (*mehlsuppenartig*)	6	Thin " faecal fluid	49
Clear watery yellow	21	Thin, pap-like brown	11
		Solid, brown	24

Hence while particularly during an epidemic clinicians may with some reason pay attention to the evacuation of rice watery stools by patients showing other features usually met with during the acute stage of severe cholera attacks, laboratory workers should beware of being influenced by the macroscopic aspect of the faecal specimens submitted to them for examination

Bacterioscopic examination

In view of the rather limited importance accorded by modern cholera workers to bacterioscopic examinations it is interesting to note that even the opinions held by the early observers regarding the value of this diagnostic method were divergent The following of these early statements deserve attention

In a report presented at the 1884 cholera conference Koch stated that he had resorted to an examination of stool smears stained with watery solutions of fuchsin or methylene blue but that in only comparatively few cases could a diagnosis be arrived at without resorting to cultivation. Occasionally however it was possible to find microscopically a number of vibrios sufficient to permit a diagnosis.

Some other early workers, of whom the first was Escherich (1884) warned against placing reliance in cholera-diagnostic work solely upon bacterioscopic examinations, particularly in view of the occurrence of spirilla which, more or less resembling *V cholerae* were specially apt to abound in the mucoid diarrhoeic stools of cholera nostras patients.

Schottelius (1885), admitted that the bacterioscopic examination of specimens taken directly from the stools of cholera-suspect individuals was apt to give doubtful or even frankly negative results. At the same time however he stressed that hanging-drop or stained preparations made after the stools had been enriched in broth proved fully satisfactory and that, therefore their microscopic examination sufficed to establish the diagnosis of cholera.

The statements made by Gruber (1887) regarding the problem at present under review though interesting, have to be interpreted with caution, not only because he examined less cholera stools than intestinal contents of supposed victims of the disease, but mainly because most of these materials had reached him with considerable delay As far as his findings go they showed that bacterioscopic examinations alone were not of decisive diagnostic importance on the one hand, several times no positive cultures could be obtained from specimens showing the presence of vibrios under the microscope, while on the other hand, in two instances *V cholerae* could be cultivated from microscopically negative specimens. However Gruber found that reliable results could be obtained if hanging-drop preparations, protected against evaporation, were kept for half an hour On account of the tendency of the vibrios to accumulate at the periphery of the drops, the re-examination of these preparations or of stained smears made from them was apt to give clear-cut results.[1]

Judging from observations made during the 1892 Hamburg outbreak Fraenkel (1892) considered cultivation a far more reliable means of diagnosing cholera than smear examination. Nevertheless the latter was apt to give frankly positive results as long as the

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[1] It was presumably on account of this early observation by Gruber that the method of examining hanging-drop preparations of cholera-suspect materials not only immediately but also after half an hour's incubation at 37°C was prescribed in the successive editions of the official German instructions for cholera-diagnostic work (see Kolle, 1904; Kolle & Schürmann, 1912; Kolle & Prigge, 1928). It was only in the last (1916) edition of these instructions, quoted by Kolle & Prigge, that the use of this method as well as examination of stained smears prepared directly from the suspect materials was made optional in all but the first cholera cases in a given locality

stools showed the rice watery appearance with mucus flocculi usually met with during the acute stage of the disease.

Revising his previous rather sceptical views, Koch (1893) in an important article on the current state of bacteriological cholera diagnosis, stated that according to the experiences recently gained in his Institute it had been possible to arrive at a positive diagnosis through mere smear examination of almost 50% of the stool specimens afterwards confirmed to contain *V. cholerae* whereas not a single instance was met with in which subsequent tests necessitated a revision of the bacterioscopically established diagnosis. Koch stressed, however, that in order to get such reliable results, adequate experience on the part of the observers was an indispensable prerequisite.

The simple technique recommended by Koch for the bacterioscopic examination of cholera-suspect stools or intestinal contents consisted of the preparation of smears on cover glasses which, after they had become air-dry, were fixed by heat and stained with diluted carbol fuchsin.[1]

Dunbar (1896) reporting on the observations he had made during the 1892 Hamburg cholera outbreak, endorsed the value of bacterioscopic examinations. He recorded that he had demonstrated the presence of cholera vibrios with this method in 60.3% of 68 patients showing manifest clinical signs of the disease and in 53.2% of 47 persons suffering from choleraic diarrhoea but only in 14.8% of 27 individuals found to be healthy carriers.

Another claim made by Dunbar was that he succeeded in arriving at a speedy diagnosis of cholera by the examination of smears made from the initially inoculated peptone water cultures after short incubation. He stated that, using this method, he was capable in 12 out of 14 instances of arriving at a positive diagnosis with the aid of peptone water cultures incubated for only 3 hours. It deserves attention, however, that Babes (1914) as well as Verzár & Weszeczky (1916), who were able to confirm their bacterioscopic findings with agglutination tests, obtained disappointing results with this rapid method.

Generally speaking, the modern experts are agreed that bacterioscopic examinations do not suffice to establish the laboratory diagnosis of cholera and that even the presumptive value of this method is limited. The main objections made to it may be summarized as follows:

1. As has been stressed by several observers recently, for instance, by Ahuja et al. (1950, 1951), the use of bacterioscopic examinations is rather redundant in that they prove most often and most frankly positive in those cases in which a presumptive diagnosis can be made on clinical grounds.

2. However, even in clinically suspicious and afterwards confirmed cholera cases bacterioscopic examination may give a negative result (see for instance, Straus & Roux, 1884; Babes, 1914; Verzár & Weszeczky, 1916; Maitra & Basu, 1924; Pollitzer, 1926).

3. In countries like India and China, where cholera-like vibrios abound in the surface-waters, the presence of these organisms in the stools not only of healthy persons, but also of individuals suffering from gastro-intestinal disturbances may prove quite misleading.

[1] Since dilutions of carbol fuchsin are not stable, a concentrated solution must be kept in stock, prepared according to Ziehl (1882) by admixing 10 ml of a saturated solution of basic fuchsin in 95% ethanol to 90 ml of a 5% aqueous solution of carbolic acid (phenol). While Koch (1893) gave no specifications in this respect, most workers resorted for the purposes of cholera laboratory diagnosis to a dilution of one part of concentrated carbol fuchsin with 9 parts of distilled water, as prescribed for instance in the official German instructions (see Koch and co-authors, 1902). However, some experts preferred more concentrated solutions, Gallut (1954), for example, recently advocating the use of a carbol fuchsin dilution of 1/5 for the purposes of cholera-diagnostic work.

4 While the cholera vibrios in stool smears do not invariably show a typical morphology other intestinal bacteria may assume an aspect more or less closely resembling that of vibrios As shown by Baerthlein (1912) and some other workers (see summary by Kolle & Prigge 1928) this is particularly true of the organism commonly known under the name of *B faecalis alcaligenes* (Petruschky 1896) met with quite frequently in work with cholera suspect stools

Though in spite of these limitations and drawbacks, the time honoured method of bacterioscopic examination is still recommended in some of the modern guides for the laboratory diagnosis of cholera (see for instance Ahuja et al 1950 1951 Gallut, 1954) the present writer cannot help wondering whether it still deserves such attention Working before and during the Second World War under rather difficult conditions in unoccupied China, he was mostly unable to use smear examination of cholera suspect stools and never had reason to assume that this omission exerted an unfavourable influence on the results of cholera laboratory diagnosis which almost always became available in less than 24 hours

Flagellar staining

The differential diagnostic importance of the methods of flagellar staining, already referred to in Chapter 3 is limited in that though some of the cholera like vibrios are distinct from *V cholerae* in possessing more than one flagellum this is by no means an invariable rule

Various methods of flagellar staining have been recommended by the different observers who have given their attention to this method (see summaries of Gruber 1894 Kolle & Gotschlich 1903 Kolle & Prigge 1928 Mackie, 1929a, and Pollitzer 1934) Since these tedious and delicate procedures can be used only for research purposes but not for the routine laboratory diagnosis of cholera it would be superfluous to enter here into a detailed description of their techniques However, it is important to add that in laboratories in which an electron microscope is available it is easily possible to reach an immediate decision whether a given vibrio possesses one or more than one flagellum

Peptone water enrichment

While as has been stated in the third chapter the idea of using fluid media for the enrichment of cholera vibrios in faecal specimens was originated by Schottelius (1885) Bujwid (1888) was the first to recommend the use of peptone water for this purpose However it was only on account of the excellent results which Dunbar obtained during the 1892 Hamburg outbreak and which he afterwards recorded in 1896 that the value of the latter eminently useful method was generally recognized As stated by Koch (1893) in a preliminary account on Dunbar's work the latter used

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line sodium carbonate to filter these solutions and to fill them in 100-ml quantities into flasks which were then sterilized Peptone water could then be easily prepared by mixing 1 part of this stock solution with 9 parts of water The mixtures were then filled in 10-ml quantities into tubes as well as in 50-ml amounts into flasks and sterilization was repeated

The following comment must be made on this method which was also prescribed in the subsequent editions of the official German instructions with the exception that the one published in 1916 and reprinted by Kolle & Prigge (1928) rendered it obligatory to use not less than 50-ml quantities of peptone water in flasks for all cholera-diagnostic work

(*a*) While Koch and co-authors specified one particular kind of peptone many other brands have been found equally suitable and some even more suitable for cholera-diagnostic work However as maintained by Kabeshima (1922) and by Bengston (1924) some kinds of peptone cannot be used for this purpose because they have a strongly acid reaction As pointed out by Bengston this untoward feature could be counteracted by a corresponding adjustment of the reaction of the media Nevertheless when one has to use a brand of peptone hitherto untried for cholera laboratory work it is well to ascertain its suitability through tests with known strains of *V cholerae*

(*b*) It will be noted that in contrast to the original recommendation of Dunham (1887) the official German instructions prescribed the use of peptone water containing 1% sodium chloride While as stated in the third chapter some authorities advised the use of a salt concentration of 3%, many workers preferred to adhere to Dunham s formula which is also given for peptone water manufacture in the standard text books on laboratory methods Gallut (1954) recommended either following this practice or utilizing peptone solutions containing 30 g of sodium chloride per litre

(*c*) The advice of Koch and co-authors (1902) to bring the peptone solutions to a suitable reaction by the incorporation of standard doses of sodium carbonate cannot be considered invariably satisfactory—not merely because this produces only a moderate alkalinity but mainly because the reaction not only of different brands of peptone but even of the water used in the various laboratories may vary considerably Modern workers therefore prefer to bring each lot of their peptone water media to a suitable pH by the easily applicable methods now generally used for the standardization of media However so far no uniform pH standard has been adopted for this purpose Thus Mackie (1929b) recommended a pH of 8.0-9 0 for peptone water manufacture and Gallut (1954) recently one of 8 6 while the investigations of Read et al (1939) referred to in Chapter 3 as well as those of Venkatraman & Ramakrishnan (1941) indicated the desirability of using a pH somewhat in excess of 9 0 As far as the present

for enrichment the weakly alkaline sterilized solution of 1% peptone and 0.5% sodium chloride originally recommended for the rapid growth of *V cholerae* by Dunham (1887) In addition to originally using this medium in test tubes for the inoculation of mucus flocculi or other small particles of the stools to be examined, Dunbar afterwards also resorted to the inoculation of larger amounts of the faecal specimens (1 ml or more) in correspondingly increased quantities of the enriching fluid in Erlenmeyer flasks This last procedure was again recommended by Abel & Claussen (1895) who advocated side by side with the tube method inoculation of 10-20-ml amounts of the faecal specimens in 5-10 times the quantity of peptone water After these fluids had been incubated for 20 hours subcultures were made from their surface in peptone water tubes and examination of the latter was continued in the usual manner

The utilization both of tubes filled with 10-ml amounts of peptone water and of flasks containing 50 ml each of this medium was also prescribed in the official instructions for cholera-diagnostic work compiled by Koch and co-authors in 1902 and promulgated in the same year However the use of the latter was restricted to the examination of specimens obtained from convalescents or supposed carriers, while in the case of the stools of cholera patients the inoculation of several (3-6) peptone water tubes was recommended. All these cultures were bacterioscopically examined after 6 hours and 12 hours incubation at 37° C and each time subcultures in peptone water tubes as well as on solid media were made from the most promising growths

In a further classical contribution to this subject, Hetsch (1903) while praising the value of peptone water enrichment, which had recently been confirmed through the large-scale investigations of Kolle & Gotschlich (1903) stressed that

"the peptone solution is by no means a medium offering selective growth conditions exclusively for the cholera vibrios but, according to their oxygen requirements and their motility all vibrio species become more less enriched on its surface" [Trans.]

Hence Hetsch continued,

"it is essential for practical diagnostic work to determine the species of the enriched vibrios through reliable and easily applicable methods of identification and for this purpose their even distribution on agar plates and the testing of the colonies thus isolated with highly specific immune sera will serve" [Trans.]

It is indeed the mode of procedure described by Hetsch, and not any rash attempt to use the primary peptone water cultures for the direct identification of *V cholerae* which constitutes the outstanding value of this and other methods of enrichment for cholera-diagnostic work.

The method of preparing peptone water for enrichment prescribed by Koch and co-authors (1902) was to make stock solutions by dissolving by heat, in 1 litre quantities of sterile distilled water 100 g of peptonum siccum Witte, 100 g of sodium chloride 1 g of potassium nitrate and 2 g of crystal

peptone water method Though the procedures recommended for this purpose in the past are not utilized any more the following among them because they attracted considerable attention for some time deserve discussion

Ottolenghi (1911) found that ox bile after alkalinization with sodium carbonate favoured the growth of *V cholerae* while inhibiting that of *E coli* and the other organisms usually met with in human faeces He therefore prepared an enrichment medium as follows

Fresh ox bile was filtered through paper and 3% of a 10% solution of crystalline sodium carbonate and 0 1% potassium nitrate were added to the filtrate The mixture was distributed in 5 ml quantities into tubes and these were sterilized in the autoclave at a pressure of ½ atmosphere

Ottolenghi recommended that for each faecal specimen 3 of these tubes should be inoculated with 1 loop 3 loops and 0 1 ml of the material under test, respectively After incubation material for bacterioscopic examination and implantation of agar plates was taken from the surface of the bile cultures.

Experimenting with pure cultures and artificially contaminated faecal specimens Ottolenghi found that his medium, while as suitable for enrichment as peptone water sometimes promoted the multiplication of *V cholerae* more slowly than the latter However owing to its inhibitory action on the other organisms the bile medium facilitated the isolation of cholera vibrios on the plates even if platings were made from the primarily inoculated tubes with considerable delay It therefore seemed particularly suitable for the examination of specimens from convalescents and suspected carriers, the vibrio content of which was apt to be scanty The bile medium like peptone water facilitated the growth of cholera like as well as of cholera vibrios

While a few workers, including Hach (1924) who worked with actual cholera stools, confirmed the good experiences of Ottolenghi others did not share these favourable opinions Thus as summarized by Kolle & Prigge (1928) Dieudonné & Baerthlein (1912) and Haendel & Baerthlein (1912) noted that Ottolenghi s method "sometimes gave better results than peptone water enrichment but that the latter was often successful where the bile method failed" Schürmann & Abelin-Rosenblat (1913) maintained in this connexion that if small inocula were used the bile medium could exert an inhibitory action on *V cholerae* as well as on other organisms. In their opinion peptone water which could be easily prepared and was stable was preferable to Ottolenghi s medium

Krombholz & Kulka (1912) while admitting that this medium exerted an inhibitory action on certain competitors of *V cholerae* occasionally met with in the stools of the patients considered it a far less suitable substrate for the growth of cholera vibrios than peptone water These two workers stressed, moreover that

"In general the solution of the problem of a maximally certain and accelerated diagnosis in cholera-suspect cases lies less *in the inhibition of the competing organisms* than in the provision of optimal conditions of growth for the cholera vibrios." [Trans.]

writer can judge the figure of 9.2, suggested by the two last mentioned groups of workers, might to advantage be adopted as standard for peptone water manufacture

(*d*) Ample experiences have shown that, when dealing with the faeces of cholera patients in the acute stage of the disease, it is quite sufficient to implant loopfuls of the specimens in tubes containing 10-ml quantities of peptone water as was originally prescribed by Koch and co-authors (1902) Certainly however when examinations have to be made of the stools of convalescents and contacts, i.e of specimens the vibrio contents of which are presumably scanty it is indicated to inoculate correspondingly larger amounts of the faeces (Koch and co-authors proposed 1 ml quantities) into flasks containing 50 ml of peptone water It is true that, if numerous examinations of this kind have to be made at the same time the use of these larger amounts of peptone water might heavily tax the resources of the laboratories. However as has been indicated earlier in this chapter this difficulty can easily be overcome and much labour can be saved at the same time, by using instead of individual faecal specimens the pooled stools of suspected cholera carriers for enrichment in peptone water or other suitable media.

As been discussed in Chapter 3 the question for how long the peptone water cultures and subcultures used for enrichment should be incubated has also not been settled It was stated that, while as a rule incubation periods of 6 hours or even more were recommended the present writer found it sufficient, when dealing with the stools of patients during out breaks, to incubate his peptone water cultures and subcultures for periods of 3 hours only The great advantage of this procedure was that, without it being necessary to arrange for a night-shift of workers, even specimens received late in the afternoon could be plated on the same day and that thus a diagnosis could as a rule be arrived at on the following morning. As noted, the precaution was taken of keeping the peptone water cultures and subcultures (from the latter of which the platings had been made) so as to make it possible to re-examine them should the original platings give an indefinite result. Actually however instances where this was necessary were exceptional

There can be no doubt, however, that in the case of specimens collected from cholera convalescents or from suspected healthy carriers the peptone water cultures and subcultures must be incubated for at least 4-5 hours, and preferably for 6 hours in the case of the primarily inoculated cultures This prolongation of the incubation time entails hardly any inconvenience, because arrangements can as a rule be made for collecting such specimens early in the day

Other enrichment methods

Several proposals have been made for using other fluid media for the enrichment of *V cholerae* in suspect materials in place of the time honoured

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Schoebl (1915) who using dry instead of fresh bile obtained unsatisfactory results with Ottolenghi's method, also maintained that bile could exert an inhibitory action on the growth of *V cholerae*

All in all it is difficult to contradict the opinion of Kolle & Prigge that there was no necessity for the adoption of Ottolenghi's method in practical cholera laboratory work

In an article which appeared in the same year as that of Ottolenghi, Kraus and co-authors (1911) reported on the suitability of a blood alkali broth for the enrichment of *V cholerae* For practical purposes they recommended the preparation of this new medium as follows

"To 100 ml of neutral broth are added 25 ml of blood alkali [prepared according to the method of Dieudonné (1909) described below]. The open flasks are kept for 3 hours at 50° and then for 24 hours at 37° and are then tubed in 5-ml quantities and inoculated." [Trans.]

Kraus & co-workers claimed that this medium was superior to peptone water as far as both the inhibition of other organisms and the enrichment of *V cholerae* were concerned.

The medium of Kraus & co-workers shared the fate of that of Ottolenghi being considered suitable by some workers and unsatisfactory by others. Among the former observers were Dieudonné & Baerthlein (1912) Haendel & Baerthlein (1912) and Schoebl (1915) Sgalitzer & Loewy (1913) even claimed that this medium, while promoting the growth of *V cholerae* exerted an inhibitory action on the cholera like vibrios. However Schürmann & Abelin-Rosenblat (1913) disapproved of the method, and their opinion was evidently shared by Kolle & Prigge Certainly account has to be taken of the tedious process of manufacturing the medium and of the inconstant results different lots may give

Goldberger (1914) made the interesting proposal to use an alkaline-egg-peptone medium for the selective enrichment of *V cholerae* As summarized by Mackie (1929b) this medium

"is composed of whole egg mixed with an equal volume of water to which mixture is added an equal volume of 5 per cent sodium carbonate the alkaline-egg preparation is finally mixed with nine parts of peptone water"

It was found that in this medium, the manufacture of which was again carefully studied by Bengston (1924) *V cholerae* multiplied less rapidly than in peptone water but continued to multiply for a longer period.

According to Mackie Goldberger's medium was "recommended as an alternative to alkaline peptone water in the Medical Research Council Special Report (1920) on the Laboratory diagnosis of intestinal infections"

A further enrichment method to be referred to at the present juncture was that of Yen (1933) who prepared for this purpose a starch-containing medium in the following manner 2 g Witte peptone, 1 g maltose, 0.5 g potassium nitrate 0 5 g crystalline sodium carbonate 10 g sodium chloride, and 0.5 g crystalline magnesium chloride are dissolved in 900 ml of distilled

water, the solution is boiled for 3 minutes and then filtered Next 100 ml of a 5% solution of soluble starch which has been previously boiled for 2 minutes, are thoroughly mixed with the filtrate The mixture is filtered through cotton and then through asbestos. After readjustment of the volume the clear filtrate is sterilized by boiling for 3 minutes and is then kept in the refrigerator Immediately before use the reaction of the medium is adjusted to pH 9 0-9.2, 5 ml of a saturated solution of phenolphthalein in 50% ethanol per litre serving as indicator The ready made medium is distributed into sterile 50-ml flasks

To utilize this medium 0 1-0 2 ml amounts of fluid or loopfuls of solid stools, were inoculated in 50-ml quantities and the flasks were incubated for 5-8 hours If a decolorization of the fluids indicated the presence of cholera or cholera like vibrios, direct agglutination tests were made with material taken from the surface of the growths. If however decolorization was incomplete or the growths were too scanty for agglutination tests 1 ml quantities were transferred to new flasks for a further incubation for 5-8 hours

Making comparative tests with his medium and with peptone water Yen found that the former better promoted the growth of *V cholerae* and restricted the growth of *E coli* than the latter However as far as these observations made with artificially contaminated faecal specimens went even the degree of inhibition produced by the starch media was by no means spectacular

The modern phase of the problem under review may be said to have started in 1939 when Read recommended the use of a modification of the bismuth-sulfite medium originally devised by Wilson & Blair (1931) for work with typhoid bacilli, for the selective enrichment of *V cholerae* The prescriptions given for the preparation of this modified medium, as brought up to date in a WHO technical report published in 1950 were as follows

Formula of the medium	*ml*
2 % peptone solution	8 8
Sea-salt mixture	1.2
Distilled water or stool suspension in distilled water	10 0
10 % mannose solution	1.0
Liquor bismuthi	0.12
20 % sodium sulfite solution	1.2
Absolute alcohol	0.2
Mercury perchloride ($HgCl_2$) 1/10 000 solution	0.8

adjusted to a pH of 9.2 with N caustic soda, with thymol blue as indicator

Formula of the sea-salt mixture	*Parts*
Sodium chloride (NaCl)	27 00
Potassium chloride (KCl)	1 00
Magnesium chloride ($MgCl_2$, $6H_2O$)	3 00
Magnesium sulfate ($MgSO_4$ $7H_2O$)	1 75
Distilled water	100 00

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found more convenient to employ a medium of double strength and to add to it an equal volume of a saline faecal emulsion "

Concerning the last paragraph of these instructions it should be noted that, for the purpose of stool examinations Read (1939) originally recommended placing the portions of his medium prepared according to the formula on page 540 into 50-ml screw-capped medicine bottles but in order to accommodate the Seitz discs or filter papers used for an improved method of water examination (see below) he recommended substituting 100-ml wide mouthed glass stoppered flasks According to him stool emulsions were prepared

" by measuring out the required volume of stool in the barrel of a Roux syringe and mixing with an equal quantity of normal saline and filtering through a single layer of lint The mixture could then be diluted as required so that the volume added to the media bottle was 10 c.c "

Commenting on the results of his laboratory observations Read maintained that

" By the use of this medium mannose fermenting vibrios can be successfully differentiated from non-mannose fermenting vibrios and from coliform types. Other common water and stool organisms except streptococci are suppressed but no method of facilitating *V cholerae* against mannose fermenting inagglutinable vibrios was discovered. The value of the method will depend on whether the mannose fermenting vibrios found in natural sources can outgrow *V cholerae* or not The difficulty due to the growth of total organisms when ordinary peptone-water enrichment is employed is overcome "

Parallel with the laboratory investigations discussed above a field trial of the new medium was undertaken by Seal (1939) who besides using the faecal samples preserved by him according to the method described earlier in this chapter (see page 527) in 10-ml quantities for inoculation in Read s fluid for the sake of comparison also inoculated 2 ml quantities into 20 ml of 1% peptone water In both cases the pH of the fluids after inoculation was readjusted to 9 2. Plates were implanted after the flasks had been incubated at 37 C overnight

Evaluating the results obtained with a total of 309 faecal specimens Seal stated that

Of the cases of clinical cholera 64 7 per cent yielded *V cholerae* in the new medium as compared to 43 1 per cent in alkaline peptone water Only 4 of 21 recent contacts gave positive results with both media."

A further important finding was that

Compared with the alkaline peptone water there was some restriction of growth of the inagglutinable vibrios in the new medium and the majority (87 4 per cent) were mannose fermenters."

The value of this method for the selective enrichment of *V cholerae* was confirmed through the laboratory studies of Wilson & Reilly (1941) and through further field work of Read & Pandit (1941)

Formula and preparation of liquor bismuthi

Bismuth citrate	60 g
Ammonia (12.5 %)	20 ml
Distilled water to make up to	500 ml

Liquor bismuthi is prepared as follows a bottle with a ground-glass stopper is filled almost completely with 500 ml of distilled water and the liquid level is marked on the side of the bottle. The water is poured out, and 60 g of bismuth citrate are introduced through a wide funnel, followed by 50 ml of distilled water The citrate is mixed with the water into a smooth paste, using a glass stirrer Next, 20 ml of 12.5 % ammonia (liquor ammonii, specific gravity 0 880) are added. The mixture is stirred with a glass rod and a chemical reaction takes place with evolution of heat. The glass stopper is inserted, the bottle shaken, and as soon as the bismuth citrate has almost entirely dissolved distilled water is added up to the 500-ml mark.

Note It should be noted that Read & Pandit (1941), while otherwise following the formula for preparing the medium, reduced the amount of liquor bismuthi to 0 04 ml.

Preparation of the medium

All ingredients are made up in separate solutions and kept in stoppered bottles. According to Read (1939) the 2 % peptone solution and the distilled water alone were autoclaved before use. The sea-salt mixture, if made up with sterile water remained sterile for practical purposes. The mannose was made up in 10 % solution for the needs of the day and was sterilized by boiling, while the sodium sulfite was merely exposed to slight heat to dissolve it.[1]

Some modifications of Read s formula were proposed by Wilson & Reilly (1940) who recommended in particular the following method for preparing a sulfite-bismuth mixture

" to avoid any variation and for convenience we have found it an advantage to use bismuth ammonio-citrate scales instead of liquor bismuthi. In the sulphite-bismuth solution there is put glucose (if we had had a supply we would have used mannose) which not only serves for the nutrition of the vibrios but also prevents oxidation of the sulphite.

" The stock solution is prepared by dissolving 20 g. sodium sulphite anhydrous in 100 c.c. of boiling water and adding to it 0 1 g. bismuth ammonio-citrate scales dissolved in 10 c.c. of water A precipitate of bismuth hydrate separates out on boiling. A solution of 20 g. of commercial glucose in 100 c.c. of boiling water is made and when cool both solutions are mixed. In place of glucose saccharose, mannitol or mannose may be employed. The stock solution keeps for months and is added to the saline peptone water just before use the *p*H of the stock solution is 9 4

" To 100 c.c. of saline peptone water *p*H 9 1 10 c.c. of stock glucose sulphite bismuth mixture are added and then 1 c.c. of absolute alcohol. We have found little advantage in the addition of 4 c.c of 1/10 000 $HgCl_2$. Wilson & Blair employed $HgCl_2$ with a view to the suppression of *Proteus* strains, but recently we have found it disappointing for this purpose. In the tubes enterococci often develop, and we have an impression that $HgCl_2$ tends to suppress them.

" In our work tubes containing 10 c.c. of the enrichment medium were inoculated with a drop of peptone water culture of the vibrio In field work it would probably be

[1] Indications for the preparation of a cheap substitute for chemically pure mannose from ivory-nut shavings (a by-product in button manufacture) have been given by Bose (1939) and in an improved form by Narayanan (1941). These substitutes were found to be suitable not only for the manufacture of the above medium but also for the preparation of solutions for fermentation tests. However as pointed out by Read & Pandit (1941), allowances in the media formula had to be made owing to the reduced concentration of mannose obtained in the rough extracts (4 per cent to 6 per cent)

devised for the examination of cholera suspect faecal specimens by Panja (1942) who found

"that when a sample of cholera stool is put into an L_3 candle fitted into a wider test tube containing peptone-water in such a position that the unglazed part of the candle is covered by the surrounding peptone water and the whole is then incubated, vibrios from the stool grow through the candle into the peptone water in 24 to 48 hours and sometimes a pure culture is obtained by this procedure in 18-20 hours. *Bact faecalis alkaligenes* motile *coliform* organisms and late lactose-fermenters also grow through the candle but not so readily as the vibrios If a small amount of the stool is mixed with peptone water and partly aspirated through the candle into the surrounding peptone-water by vacuum action, growth of the vibrios occurs earlier (18 to 20 hours)"

Panja further established that the value of this method for the isolation of *V cholerae* was greatly enhanced if in order to counteract the growth of coliform organisms, boric acid in a strength of 0 08% was added to the peptone water and the final pH was adjusted to 9 0

The great value of Panja s "candle boric peptone water" method was demonstrated by tests with 45 stool samples collected during the declining period of an epidemic from patients showing clinical signs of cholera while direct plating of these specimens on bile salt agar gave only 44% positive results, *V cholerae* was isolated with the aid of Panja s method in 87%. Further as will be recorded in a later section of this chapter Panja s method of cultivation also yielded considerably more positive results than direct plating of cholera suspect faecal specimens on a bismuth sulfite medium.

However impressive though these results are account has to be taken of the great difficulties which would arise with the large scale use of Panja s method under field conditions He himself pointed out in this connexion that

"To ensure success candles should be tested before sterilization for patent porosity and leakage by forcing air under pressure of 15 lb to 20 lb [1.05 to 1 40 kg per cm²] above the atmospheric pressure, while the candles are immersed in water If no air passes through, blockage is indicated and if large bubbles come out there is leakage."

It is well nigh impossible to see how in places where no first-class laboratory facilities are available this desideratum could be fulfilled and how under such circumstances the candles could be adequately sterilized without damaging them It is therefore not surprising to find that, in spite of its excellent record, Panja s method was not mentioned in the instructions for cholera laboratory work compiled by a group of the leading Indian cholera experts (see Ahuja et al 1950 1951)

Ch i & Zia (1949) tried to combine the enriching properties of the fluid medium of Yen (1933—see page 538) and of potassium tellurite in the following way

A broth base was prepared by adding 5 g of beef extract 10 g of peptone 8 g of sodium chloride, and 1 g each of potassium nitrate and of magnesium chloride ($MgCl_2$,

Wilson & Reilly (1941) found that—in contrast to 31 strains of cholera vibrios, which developed rapidly and profusely in their medium—only 6 out of 25 cholera like and "paracholera" strains grew well, and 19 but scantily or not at all Out of 11 El Tor strains 5 only grew in the fluid bismuth-sulfite medium.

Recording the results of their field investigations, Read & Pandit (1941) stated that they had found

"the non-haemolytic agglutinable vibrio in all except one of the clinical cases in areas where the presence of cholera could be established, provided the examination was carried out sufficiently early in the disease"

It is further noteworthy that Venkatraman (1949) used the enrichment method described above with evident success for the detection of *V cholerae* in pooled stool samples which had been collected from the staff members of a cholera hospital (see page 530 above)

In a series of articles which began to appear two years after Read's initial publication Gohar (1941 1951) and Gohar & Makkawi (1947 1948a, 1948b) recommended a medium containing potassium tellurite for the selective enrichment of *V cholerae* The technique finally adopted for this purpose was thus described by Gohar & Makkawi (1948b) and by Gohar (1951)

Whenever the medium was required, an aqueous solution containing 1 % peptone and 0.5 % NaCl was made, and enough sodium carbonate (usually 0.2 %) was added to obtain a pH of 9 0 0.5 % sodium taurocholate was added and the medium was distributed into flasks, preferably 25-ml conical flasks, which were filled to the bottom of the neck so as to obtain a comparatively small surface and thus to concentrate the cholera vibrios growing in the medium.

If possible 3 such flasks were provided for each faecal specimen, to which enough potassium tellurite was added to obtain concentrations of 1/100 000 1/120 000 and 1/400 000 respectively However if economy was essential, the medium was distributed in 10-ml quantities into tubes, and 0 002 % of potassium tellurite was inserted to produce a concentration of 1/200 000 This usually sufficed to inhibit the growth of *E. coli*, while the sodium taurocholate suppressed that of coccal forms and most of the anthracoids met with in the stool specimens.

The flasks or tubes were heavily inoculated with the stools and incubated at 37° C loopfuls being taken from the surface after 8 and again after 24 hours with the help of a *loop bent at right angles, so that it was level with the surface when touching* the medium. The material thus taken was implanted into a special semi-solid agar containing 1 % mannite and 0 1 % glucose with Andrade's indicator (see Gohar 1947 1948).

Testing several other chemicals and dyes besides potassium tellurite for their suitability in inhibiting the growth of the usual intestinal organisms without interfering with that of *V cholerae* Gohar & Makkawi (1947) found potassium and sodium selenite in dilutions of 1/500 to 1/1000 of limited usefulness in this respect Neutral red and other dyes, on the other hand, were found to exert an inimical action on the vibrios rather than on *E. coli* and *B faecalis alcaligenes*

Before dealing with a further potassium tellurite medium recommended by Chi & Zia (1949) attention has to be drawn to a peculiar method

devised for the examination of cholera suspect faecal specimens by Panja (1942), who found

"that when a sample of cholera stool is put into an L_4 candle fitted into a wider test tube containing peptone-water in such a position that the unglazed part of the candle is covered by the surrounding peptone-water and the whole is then incubated, vibrios from the stool grow through the candle into the peptone water in 24 to 48 hours and sometimes a pure culture is obtained by this procedure in 18-20 hours *Bact faecalis alkaligenes* motile *coliform* organisms and late lactose-fermenters also grow through the candle but not so readily as the vibrios If a small amount of the stool is mixed with peptone water and partly aspirated through the candle into the surrounding peptone-water by vacuum action, growth of the vibrios occurs earlier (18 to 20 hours)"

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$6H_2O$) to 900 ml of distilled water After this mixture had been heated to solution, it was added to a solution of 5 g of soluble starch in 100 ml of water which had been boiled for 2 minutes.

The pH of the fluid was next adjusted to 9.2 with 10 N caustic soda, using 0.04% thymol blue as indicator or with the aid of a potentiometer The medium was then distributed into flasks in 100-ml quantities and sterilized in the autoclave at 12 pounds per square inch (0.84 kg per cm^2) for 20 minutes.

Immediately before use 1 ml each of a sterilized 0.2% aqueous solution of potassium tellurite and of a 0.5% solution of rosolic acid in 90% ethanol were added to each flask. After thorough mixing, the medium was then distributed in 10-ml amounts into sterilized tubes.

To test the medium, the tubes were inoculated with drops of artificially cholera-contaminated stool emulsions. After an incubation of from 10 to 24 hours the tubes were inspected and those showing decolorization were tested with Gram's iodine solution. The growths giving a negative starch reaction were then used for slide agglutination tests and for plating.

While the results obtained with this new medium in the laboratory were most satisfactory, Chi & Zia stressed that

"while absence of decolorization and positive starch test have invariably indicated the absence of *V cholerae* in the specimens, simple decolorization and negative test did not always indicate growth [of this organism]. It appeared to us that certain as yet unknown enzymes probably acted on the starch to produce this false effect. For that reason the medium must be either freshly prepared before use or it must be stored in flasks with rubber stoppers before its final preparation "

The two workers added that

"In view of the inhibitory action of rosolic acid and potassium tellurite, it may be possible to plant larger inoculum with feces from suspected carriers or with infected water In that case, a double strength liquid medium may be employed and equal volume of inoculum could be used. The possibility of recovering the infecting organism may thus be greatly increased."

However one cannot help feeling that the rather complicated procedure involved would limit the usefulness of this method, even if it should be found as satisfactory in the field as in laboratory tests

While trying to devise a solid medium suitable for the identification of *V cholerae* Dishon (1951) also established that a fluid medium prepared on similar lines was useful for the selective enrichment of this organism The fluid medium was obtained by adding the following ingredients to 100-ml quantities of a broth base containing 0.6% meat extract, 0.5% bacto-peptone and 2% NaCl

10% sodium sulfite solution, 1.5 ml 20% sodium carbonate solution, 4.0 ml 20% saccharose solution, 2.5 ml saturated alcoholic solution of acid fuchsin, 0.2 ml as well as gentian violet and brilliant green to a concentration of 1/200 000.

Dishon stated that his fluid medium

"inhibits the growth of Coliforms, spore formers and Pseudomonas for 12 hours, and of Cocci for 24 hours, whilst the pellicle of the *Vibrio cholerae* appears after the first 6 hours "

In comparative tests made with artificially contaminated faecal specimens, it was found that cholera vibrios could be isolated with the aid of Dishon's fluid medium when present in the stool samples in a dilution of 10^{-9} whereas in the case of alkaline peptone water the highest dilution from which positive results could be obtained was 10^{-7}. It deserves attention however, that the peptone water used for these tests had a pH of only 8.5-8.7.

For practical purposes Dishon recommended adding 1 ml of the test specimens to 10 ml of the fluid medium, incubating for 6 hours at 37° C and then taking material for plating besides making stained smears from the pellicle.

A comparative study of the suitability of various fluid media for the growth of *V. cholerae* in the presence of other organisms with which it has to compete under normal conditions was made by Felsenfeld et al. (1951). Details of the technique they used for this purpose were as follows:

The media used in 100-ml quantities were (1) the preserving fluid of Venkatraman & Ramakrishnan (1941), (2) Wilson & Reilly's (1940) modification of the bismuth-sulfite medium, prepared by dissolving 20 g anhydrous sodium sulfite in 100 ml boiling distilled water, adding 10 ml of a 1 solution of iron ammonium sulfate "green scales" and 100 ml of a 20% glucose solution and mixing 10 ml of this stock solution with 100 ml of peptone water[1] containing 1% peptone and 2% NaCl, pH 9.2, (3) Gohar's (1948) medium, made up by adding 0.2 ml of a 1 aqueous solution of potassium tellurite to 100 ml of peptone water pH 7.8-8.0, (4) alkaline peptone water containing 1 peptone and 0.5 NaCl, (5) alkaline selenite-F broth "prepared by adding enough 10 per cent aqueous sodium carbonate solution to Selenite-F to bring the pH of the fluid to the desired alkalinity" (see tabulation below).[2]

The inocula used for these tests consisted of (a) 500-1000 cholera vibrios per ml of the media and (*b*) 10 000-15 000 organisms each of *E. coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and an enterococcus strain.

The results of the tests made according to this technique with 53 cholera strains were summarized by Felsenfeld et al. in the form of the following table showing the average multiplication rate of cholera vibrios in fluid media inoculated with bacterial mixtures (as specified above):

Medium	*Vibrio count in millions per ml medium after incubation*			
	At 37° C		*At 22°-26° C*	
	24 hours	*48 hours*	*24 hours*	*48 hours*
Venkatraman & Ramakrishnan	100	420	30	250
Wilson & Reilly	70	90	25	30
Gohar	380	800	250	370
Alkaline peptone "broth" (water)	400	880	220	680
Selenite broth pH 6.9 to 7.1	150	75	40	45
Selenite broth pH 7.4 to 7.6	270	310	130	160
Selenite broth pH 7.8 to 8.0	330	570	180	240

[1] Though Felsenfeld et al. spoke at this juncture and also elsewhere in their article of "peptone broth" they obviously referred to what is called peptone water in the present study.

[2] The commercial selenite-F product used by Felsenfeld et al. was undoubtedly similar to, if not identical with, the "selenite F broth", prepared according to the handbook, *Diagnostic Procedures and Reagents*, issued by the American Public Health Association (1950), by dissolving 5 g peptone, 4 g lactose, 10 g anhydrous sodium phosphate and 4 g sodium acid selenite in 1 litre of water.

$6H_2O$) to 900 ml of distilled water After this mixture had been heated to solution, it was added to a solution of 5 g of soluble starch in 100 ml of water which had been boiled for 2 minutes.

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disproved (see for instance Kolle 1903 Kolle & Gotschlich 1903 Hetsch 1903)

While admitting that a practised observer could also distinguish the cholera vibrios developing on agar plates from the usual faecal and water bacteria Koch considered it necessary to verify the vibrio nature of such suspect colonies through bacterioscopic examinations However the great advantage of agar cultures was that they could be incubated at 37° C and therefore in contrast to the gelatin plates yielded colonies of a size suitable for identification tests after as little as 8-10 hours. Koch pointed out however that in order to obtain such satisfactory results it was necessary (*a*) to resort to preliminary enrichment in peptone water (*b*) to spread out the material to be examined on the surface of the plates instead of admixing it to the liquefied agar and (*c*) to use agar plates with a dry surface obtained by keeping them for a few days in the incubator before inoculation.[1]

As has already been described in the fourth chapter the well nigh ritualistic scheme for cholera diagnosis painstakingly built up by Koch was soon swept aside by the tide of epochal discoveries in the field of immunology which led to the introduction of far more reliable serological methods for the identification of *V cholerae* This new orientation of cholera-diagnostic work was fully recognized in the regulations framed by Koch and co-authors (1902) for official use While cultivation on gelatin as well as on agar plates was still made obligatory in these instructions, it was recommended that the pure subcultures necessary for identification tests be obtained from agar plates after an incubation ranging from 12 to 18 hours

The method of preparing the gelatin media was thus described in these instructions

(1) A meat-peptone broth was prepared by (*a*) digesting fat-free minced beef in water for 24 hours in the cold (or for 1 hour at 37° C) at a ratio of ½ kg of the meat to 1 litre of water (*b*) squeezing out the juice of the beef through a cloth (*c*) adding per litre of the fluid thus obtained 10 g of peptone and 5 g NaCl (*d*) boiling for ½ hour· (*e*) rendering the reaction alkaline with sodium carbonate solution (*f*) again boiling for ¾ hour· and (*g*) filtering.

(2) To prepare the medium, 1 litre of this broth was added to 100 g of gelatin, the latter dissolved by gentle heating, and the fluid rendered neutral to litmus and then alkaline by the addition of 3 ml of a 10% solution of crystalline sodium carbonate per 100 ml. The medium was then heated in the steam sterilizer for ¾ hour and filtered.

Similarly the method of agar preparation prescribed in the German instructions was to add 30 g of agar per litre of the neutral broth described above to boil until this was dissolved, to neutralize again, and to add 3 ml of a 10% solution of crystalline sodium carbonate to each 100 ml The alkalinized medium was again boiled for ¾ hour filtered, distributed

[1] Koch and co-authors (1902) stated that incubation of the plates for half an hour was sufficient, provided that the dishes were kept open during this time in an inverted position.

It will be noted that in these laboratory tests the modified bismuth-sulfite medium gave results far below those obtained by enrichment in the other media However as has been discussed above the efficacy of the bismuth-sulfite medium for the selective enrichment of *V cholerae* under actual conditions has been amply proven so that there is no reason to doubt its full adequacy for practical cholera laboratory work. At the same time, however it would seem indicated to compare its efficacy in diagnostic work with that of the potassium tellurite medium, because the latter if equally reliable would be preferable on account of the simplicity of its preparation

Cultivation on solid media

The method of cultivating cholera suspect stools on solid media originally recommended by Koch (1884) was as follows

"A very small mucus floccule is put into 10 cc nutrient gelatin [meat-infusion peptone gelatin with a gelatin content of 10 / and a weakly alkaline reaction] and is distributed by moving the fluid. One then pours the fluid gelatin on a horizontal glass plate, which is kept cooled by putting ice under it. If spread out with a sterile glass rod, the gelatin very rapidly solidifies. The plate is kept moist under a glass bell until the bacterial colonies develop and is then examined with a suitable magnification of the microscope." [Trans.]

As Koch described in great detail, the growth appearances of *V cholerae* on the gelatin plates, and more still those becoming manifest when gelatin stab subcultures were made from the cholera suspect colonies developing on the plates, seemed to be so characteristic as to permit a distinction of this organism from all other bacterial species. On the other hand, though briefly mentioning that the cholera vibrios could be grown also on "agar-agar" plates, Koch evidently placed no confidence in this method of cultivation.

Again dealing with the methods of cholera laboratory diagnosis in 1893 Koch no longer upheld the diagnostic importance of gelatin stab-cultures, but still stressed that of gelatin plates, particularly if this mode of cultivation was used in combination with peptone water enrichment. He emphasized, however that in order to obtain reliable results, the gelatin plates (made by then with the aid of dishes devised by Petri, 1887) had to be kept at or quite near 22° C because only under these circumstances did the cholera colonies, after an incubation of 15-20 hours, assume their characteristic aspect If he continued,

"the cultures are kept at too high a temperature, or if the gelatin is unsuitable and becomes too soft at 22° then the cholera colonies liquefy the gelatin to a greater extent, and thus assume an aspect closely resembling that of Finkler s bacteria" [Trans.]

It will be noted that Koch thus still stuck to the belief that the growth appearances of *V cholerae* on gelatin plates were different from those of the cholera like vibrios—a belief which not long afterwards was definitely

stools Hirschbruch & Schwer pointed out that many cholera like vibrios reacted on this medium like *V cholerae* Identification tests were therefore indispensable For the latter purpose they recommended the use of hanging drop preparations for preliminary agglutination tests and confirmation of positive results thus obtained by Pfeiffer s reaction

In a subsequent paper Hirschbruch & Schwer (1904) advocated the use instead of litmus solution, of azolitmin in a proportion of 0 4 g per litre of agar for the preparation of their medium. The suitability of modified Drigalski-Conradi media for the isolation of cholera and cholera like vibrios was confirmed by Klein (1905) Subsequently Rivas & Smith (1912) advised direct cultivation of cholera suspect faecal specimens on lactose litmus agar while Stokes & Hachtel (1913) recommended the use of an azolitmin-containing lactose glycerol agar for the isolation of *V cholerae* Aronson (1915) asserted in contrast to these workers that, owing to the fermentation of lactose produced by this organism it grew on Drigalski Conradi plates in the form of reddish instead of blue colonies

Further solid media for the selective cultivation of *V cholerae* devised from 1909 onwards by a considerable number of workers may be classified as follows

1 Media prepared with blood or blood derivatives

As already mentioned Dieudonné in 1909 introduced a blood alkali agar for the selective cultivation of *V cholerae* describing the manufacture of this medium as follows

"If one adds to defibrinated cattle blood equal parts of normal caustic potash, a laked blood-alkali solution is formed which can be sterilized in the steam-sterilizer If one adds 30 parts of this solution to 70 parts of ordinary litmus-neutral agar [one obtains a medium] on which the cholera vibrios grow abundantly whereas *B coli* develops not at all or only very scantily" [Trans.]

Dieudonné pointed out that it was necessary to dry the plates poured with this medium for several days at 37° C or for 5 minutes at 60° C. While plates implanted with normal stools showed no bacterial growth at all *V cholerae* could be isolated from those inoculated with artificially cholera contaminated faecal specimens The agglutinability of the cholera vibrios thus cultivated was not impaired in comparison with that of agar grown vibrios—an observation which in spite of some statements to the contrary was confirmed by the majority of the subsequent workers.

While Dieudonné in his short note gave no description of the growth appearances of the cholera vibrios on his medium Huntemüller (1909) stated in an article published at the same time that the organisms developed on this medium in the form of large circular colonies with entire edges, showing a glassy transparency in transmitted light, but appearing greyish in reflected light

in flasks or tubes and sterilized repeatedly in the steam sterilizer (or according to the present practice once in the autoclave)

As in the case of peptone water manufacture modern workers, instead of using standard doses of sodium carbonate solutions to bring the solid media for cholera-diagnostic work to an adequate degree of alkalinity, will prefer to determine the pH and to bring this to a proper standard by the addition of the amount of a suitable alkali required for each lot of the media in preparation A pH above 9 0—e g. one of 9.2—is desirable in the case of the solid plain media as well as in that of peptone water

Though the problem of preparing solid media selectively suitable for the isolation of *V cholerae* began to attract much attention only after Dieudonné had proposed such a medium in 1909 this question had already been considered by several earlier workers. Some of them for instance, Dahmen (1892) and Fraenkel (1892) stressed in this connexion the importance of using, instead of the weakly alkaline gelatin originally resorted to by Koch (1884) a gelatin with a stronger alkalinity Deycke (1893) recommended for the same purpose an alkali albuminate gelatin, while Dunbar (1896) worked not only with the usual gelatin but also with soda gelatin plates

In view of the ample use afterwards made of blood and blood derivatives for the manufacture of cholera-selective media, it is interesting to note that Heim (1901) devised "blood-decoction" (*Blutdekokt*) media for the isolation of *V cholerae* To prepare these in solid form he added gelatin or agar to the fluids obtained by (*a*) boiling the coagulum of cattle, horse, or pig blood, mixed with equal parts of water in the steam sterilizer (*b*) squeezing out the juice through a cloth and (*c*) filtering. The resulting liquid was weakly alkaline in reaction

The use of the medium originally introduced by Drigalski & Conradi (1902) for the isolation of *S typhosa* for cholera-diagnostic work was recommended by Hirschbruch & Schwer (1903) The modified method utilized by the last mentioned two workers for preparing this medium was as follows

20 g agar 10 g Liebig s meat extract, 10 g peptone, and 5 g NaCl are boiled in 1 litre of tap-water for 1½ hours and, after filtration, for a further 30 minutes. After addition of 15 g lactose and boiling for 15 minutes, the reaction is rendered alkaline with the aid of a sterilized aqueous solution of sodium carbonate. One adds then 130 ml litmus solution (Kubel Tiemann) which has been boiled for ¼ hour as well as 10 ml of a solution of crystal-violet B in 100 ml hot distilled and sterilized water After thorough shaking, 8-ml amounts of the medium are used to pour plates, which are kept uncovered for about ½ hour until they have cooled and solidified.

Hirschbruch & Schwer stated that, in contrast to the colonies of *E coli* which, when grown on this medium were red and surrounded by a red zone, cholera colonies, becoming well developed after an incubation of 10-20 hours, were blue and surrounded by a blue zone

Commenting upon ample tests they had made with different pure cultures and also with artificially cholera-contaminated specimens of diarrhoeic

Nevertheless, on account of the objections mentioned above and of others, during the years immediately following Dieudonné's publication several modifications of his method and several substitute selective media were recommended. The following among these alternative procedures deserve particular mention.

(a) *Modifications of Dieudonné's method.* In a valuable study on cholera selective media Neufeld & Woithe (1910) recommended that, immediately before the plates were poured, 2 ml of 10% lactic acid should be added to each 100 ml of Dieudonné's blood alkali agar medium in order to counteract ammonia formation and to neutralize any excess of alkali that might be present. The two workers found that plates made with this modified medium became ready for use as soon as they had been dried for half an hour at 60°C. However, in contrast to ordinary Dieudonné plates which remained fit for use for a week (see Weisskopf, 1911), those prepared according to Neufeld & Woithe's method remained selective for 24-28 hours only, because their alkalinity rapidly decreased.

Pilon (1911) claimed that freshly poured Dieudonné plates could be made fit for use by keeping them for about one hour in a carbon-dioxide atmosphere. He explained this result by the assumption that the unsuitability of such plates immediately after pouring was due to the presence of an excess of free caustic potash and not to that of ammonia as maintained by most observers. In a CO_2 atmosphere the caustic potash was rapidly converted into potassium carbonate, whereas in the case of the plates exposed to air it took about 24 hours before this conversion reached a sufficient degree to render the medium fit for use.

Another way to hasten the maturing of Dieudonné plates was according to Moldovan (1912) to admix, when preparing the medium, only one part of the blood-alkali mixture to 4 parts of agar. He claimed that plates made from this modified medium could be used after only 6 hours, i.e. at the time when the peptone water cultures inoculated with cholera suspect stools had become ready for subcultivation. However Haendel & Baerthlein (1912) found that the media prepared in this manner as well as those made according to Neufeld & Woithe's method were less reliable than the Dieudonné plates, occasionally inhibiting the growth of *V. cholerae*.

According to Hall (1916) it was essential to store the blood alkali mixtures for 6-8 weeks in flasks plugged with cotton wool, because plates prepared with such stock material were immediately ready for use. More important still was the assertion of Mackie (1929b)

> that by repeated steaming of the blood-alkali until the ammoniacal odour is removed, the completed [Dieudonné] medium prepared from it can be used immediately and the blood-alkali can be kept for considerable periods without losing its selective properties."

Studying possibilities of improving Dieudonné's medium, Hofer & Hovorka (1913) paid particular attention to the fact that this, while inhibiting the growth of most intestinal bacteria, exerted no such action on some

Huntemüller also gave the following more detailed indications for the preparation of the new medium

The cattle blood was collected in sterile glass jars containing glass beads, was defibrinated by shaking for ½ hour and, after the addition of equal amounts of normal caustic potash, steam-sterilized for ½ hour This blood-alkali solution, which kept indefinitely was added, whenever needed, to neutral agar in a proportion of 3 7 and plates were poured forthwith and dried for ½ hour at 60°C.

Huntemüller added that it was indispensable to keep the plates for at least 24 hours at room temperature before they were used so that the con siderable amounts of ammonia which were at first given off by the medium and which inhibited the growth of *V cholerae* could evaporate

While confirming that Dieudonné s medium inhibited the growth of *E coli* Huntemüller found that it promoted the growth of cholera like as well as of cholera vibrios and also noted in one instance that the medium though considerably restricting the growth of *Ps pyocyanea* did not totally inhibit it Nevertheless, the results obtained by Huntemüller in ample tests with a large number of cholera strains were so satisfactory that it was decided to use Dieudonné s medium side by side with alkaline agar for the examination of cholera suspect stools in the Berlin Institute for Infectious Diseases headed by Gaffky

The initial experiences of Dieudonné and of Huntemülller stimulated many further observations recorded in numerous publications While admitting the great progress made in cholera-diagnostic work through the introduction of Dieudonné s medium a considerable number of the subsequent observers pointed out that it was by no means fully satisfactory—mainly on account of the necessity of procuring fresh cattle blood for its manufacture and of the delay of at least 24 hours caused by the need for letting the plates mature. It thus appeared that it was impossible to take advantage of the new medium under the conditions where its use would have been most desirable—namely when dealing with cholera suspect stools in localities hitherto free from the infection.

It is important to note that neither of these main criticisms levelled against Dieudonné s method was fully justified As Huntemüller had noted, the blood alkali mixture was rather stable and thus could quite easily be kept in stock to serve in emergencies in places threatened by cholera. Further as shown by observations recorded by Haendel & Baerthlein (1912) it was even possible to exsiccate Dieudonné s medium after plates had been poured, the resulting coarse brown powder keeping well if protected against moisture and being reconditionable at short notice Moreover as found, in 1909 by Hachla & Holobut, it was not indispensable to use cattle blood for manufacturing the medium, pig or horse blood proving even more satisfactory for this purpose Finally as will be further discussed below several methods were recommended for rendering the Dieudonné plates usable within a short time of being made

of even the alkali tolerant *Ps pyocyanea* to a degree sufficient for the practical purposes of cholera diagnosis

According to Takano and co-workers (1926) a similar medium was also prepared by Tokunaga (1911)

Pilon (1911) stressed that as had been shown by Deeleman (1897) the cholera vibrios were endowed not so much with a tolerance to caustic potash or caustic soda as with a tolerance to carbonates. Pilon accordingly recommended preparing a medium for the selective cultivation of *V cholerae* as follows defibrinated blood of pigs goats or rabbits is mixed with an equal amount of a 12% solution of crystalline sodium carbonate and 3 parts of this mixture are added to 7 parts of neutral 4% agar after thorough mixing the blood/sodium-carbonate agar is poured into Petri dishes which are left open until solidification has taken place the plates become fit for use after 30-45 minutes.

Following Esch s method but using a different haemoglobin product Kabeshima (1913) prepared a selective medium for cholera-diagnostic work by (*a*) adding to 80 ml of 3% neutral agar, which had been liquefied in the steam-sterilizer 10 ml of an 18% solution of sodium carbonate (*b*) boiling the mixture for about 10 minutes (*c*) adding, after the agar had been cooled to about 50 C, 3 g of a commercial haemoglobin extract and (*d*) after thorough mixing, using this material for pouring 7 plates, which were left uncovered until the agar had solidified The plates were immediately fit for use but it was well to dry them first by keeping them for 20-30 minutes open and in an inverted position in the incubator

A medium similar to that of Kabeshima was recommended by Baerthlein & Gildemeister (1915)

An exhaustive study on the comparative value of Dieudonné s and the above mentioned substitute media—with the exception of that of Esch which in the experience of Haendel & Baerthlein (1912) showed an unsatisfactory degree of selectivity [1]—conducted by Baerthlein & Gildemeister (1915) with over 100 stools of cholera patients or carriers, led to the following main conclusions

(1) Pure cultures of *V cholerae* grew most abundantly (*üppig*) within 16-18 hours on Kabeshima s medium and vigorously on Dieudonné s and Pilon s media.

(2) Pure cultures of cholera like vibrios invariably grew on these three media though less well than cholera vibrios

(3) Pure cultures of *B faecalis alcaligenes* usually grew abundantly on Dieudonné s and Pilon s media but scantily on Kabeshima s medium. *Ps pyocyanea* grew almost invariably on these three media but developed scantily while pure cultures of *Proteus* grew but exceptionally

[1] It is important to note that, no doubt in view of the favourable experiences recorded by some other observers, the 1916 German instructions for cholera diagnostic work (see Kolle & Prigge 1928 p. 81) recommended the use of Esch s medium if no suitable Dieudonné plates were available.

organisms especially the *B faecalis alcaligenes* (see Glaser & Hachla, 1911) To enhance this incomplete selectivity Hofer & Hovorka prepared a modified medium by (*a*) adding 4 ml of cattle blood to 16 ml of normal caustic potash (*b*) incorporating this blood alkali mixture into 80 ml of freshly prepared 3% neutral agar and (*c*) then adding per 10 ml of the medium 0 5 ml of a 0 1% solution of crystal violet in distilled water To become ready for use, the poured plates had to be kept partly open in the incubator for 24 hours and after closing, for a further 12 hours at room temperature.

Experimenting with pure cultures of cholera and cholera like vibrios as well as of *B faecalis alcaligenes Proteus* etc and with mixed peptone water cultures of these organisms, Hofer & Hovorka found that their modified medium effectively inhibited not only the growth of the bacillary species but also that of a number of cholera like strains.

These favourable experiences were confirmed by Fülgner (1914) However Baerthlein & Gildemeister (1915) while admitting the high selectivity of Hofer & Hovorka s medium, found that it very often impeded the growth of *V cholerae* as well.

Lentz (1915), obtaining unsatisfactory results in field work with Dieudonné media which had been kept in stock in dry form (but had apparently not been stored in a proper manner) recommended drying the blood alkali mixture separately and admixing the resulting powder after solution in distilled water to the agar immediately before plates had to be prepared He found that such media could be used at once and continued to give fully satisfactory results for 8-10 days

While confirming the value of Lentz s method, Fürst (1916) found it advantageous to use instead of distilled water a 0 3% solution of sodium carbonate for dissolving the blood alkali powder and to employ an agar containing 2% of cane sugar

Finally reference has to be made at the present juncture to the recommendation by Ghedini (1916) to enhance the selectivity of Dieudonné media by (*a*) titrating the blood alkali solution and adding further alkali if necessary and (*b*) using an alkaline, instead of a neutral agar

(*b*) *Substitute selective media* Esch (1910) recommended that, instead of fresh cattle blood, dry haemoglobin, as commonly prepared from horse blood, should be used for the manufacture of blood-alkali media A 5-g quantity of this substance was dissolved in 15 ml of normal caustic soda + 15 ml of distilled water and, after the solution had been sterilized in the steam sterilizer for 1 hour 15-ml amounts of it were added to 85-ml quantities of neutral agar Esch (1910 1912) stated that plates poured with this medium which could be used after they had been dried at room temperature for 1 hour were as satisfactory for the cultivation of *V cholerae* as Dieudonné s original medium Further though not exerting such marked inhibitory action on other organisms, Esch s medium retarded the growth

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(4) Inoculation of faecal specimens of cholera patients or carriers invariably gave a positive result in the case of Dieudonné's and Kabeshima's media, while Pilon's medium failed in some instances

(5) When such faecal specimens were used pure cultures of *V cholerae* resulted in 54 7% of plates with Kabeshima's medium in 55 6% of Dieudonné and 57 5% of Pilon plates. Among the contaminants *B faecalis alcaligenes* was met with in 25 7% of the Dieudonné plates, in 26 6% of Pilon plates, but in only 4 75% of the Kabeshima plates. While poorly developed colonies of enterococci were invariably present on all three media, colonies of *Proteus Ps pyocyanea* and dysentery bacilli were met with but exceptionally

(6) While the value of Dieudonné's original method for the isolation of *V cholerae* had thus been demonstrated once more it had to be stressed that this medium could not be used immediately and often did not sufficiently suppress the growth of *B faecalis alcaligenes*

(7) Pilon's medium, while immediately fit for use, was in general not inferior to that of Dieudonné but did fail occasionally

(8) Apart from being highly selective and suitable for the cultivation of *V cholerae* within 12 16 hours, Kabeshima's medium also had the advantages of (*a*) the commercial availability of its ingredients (*b*) a simple method of preparation (*c*) immediate readiness and (*d*) marked inhibition of *B faecalis alcaligenes* However this medium did not keep well and its alkalinity was apt to vary with the result that the growth of cholera vibrios was sometimes markedly impeded or even inhibited.

(9) To overcome these drawbacks Baerthlein & Gildemeister advocated (*a*) sterilizing the haemoglobin extract through boiling in caustic potash and (*b*) resorting, as far as necessary to further alkalinization with a 5.5% solution of anhydrous sodium carbonate (*Sodamehl*) This modified medium kept for two weeks and ensured a constantly good growth of *V cholerae* Its selectivity was also satisfactory an occasionally more marked growth of *B faecalis alcaligenes* never interfering with the rapid isolation of the cholera vibrios

Whatever the merits of the substitute media were both before and during the First World War Dieudonné's method was by far the most frequently used in actual laboratory practice by Bürgers (1910) and a considerable number of subsequent workers in Europe as well as by Greig (1913 1917) in India, with equally satisfactory results A large-scale study undertaken during the 1930 cholera outbreak in co-operation with the present writer by the Shanghai Public Health Laboratory (see Pollitzer 1934) also confirmed the value of Dieudonné's method. Still, while parallel tests made with alkaline agar almost invariably gave positive results, absence of growth on the Dieudonné plates inoculated with the same cholera stools or peptone water subcultures, though rare was not excep-

tional The practical recommendations made on account of these experiences were that in laboratory work with actual cholera stools it was indispensable (*a*) to use cultivation on plain alkaline agar side by side with that on Dieudonné plates, and (*b*) to ascertain the proper ripeness of the latter through continuous controls with known *V cholerae* strains

Interest in the use of blood alkali media in cholera laboratory work was revived through profound investigations undertaken by Vedder & van Dam (1932a, 1932b) Studying the reasons why the Dieudonné plates at first inhibited all bacterial growth and after they had selectively promoted for some time the growth of *V cholerae* they lost their selectivity Vedder & van Dam (1932a) came to the conclusion that in the case of this medium

"ripeness and selectivity are connected with a definite pH range which lies between about 9 0 to 9 6 At lower values other organisms also grow and therefore the plate is no longer selective. At higher values the cholera vibrio also does not grow The ripening of the Dieudonné medium is due therefore to a decrease of the pH value caused mainly by an entry of CO_2 from the air but partly also by an elimination [*Austritt*] of NH_3 from the plate Differences in ripeness and selectivity of different Dieudonné fluids and series of plates as well as the variations in the ripeness and selectivity of one and the same fluid at different times are connected with differences in the pH values" [Trans.]

On the basis of these and further studies Vedder & van Dam (1932b) recommended preparing the following two media for cholera diagnostic work

(1) 1 g haemoglobin is boiled for a few minutes in 20 ml of 0.2 N caustic potash. After the fluid has been rapidly cooled, one adds 120 mg glycocoll and, after a few minutes, 80 ml of liquefied but not too hot agar made from 1 g peptone, 0.5 g sodium chloride, and 3 g agar per 100 ml of water The 6 plates poured from this material are immediately fit for use without preliminary drying in the incubator but lose their selectivity if kept in storage.

(2) 1 g haemoglobin is triturated in a mortar with 4 ml K_2CO_3 (prepared by dissolving 6 9 g anhydrous potassium carbonate in 100 ml boiled distilled water) One then adds (*a*) 1 ml of $KHCO_3$ solution (prepared by dissolving 5 g of hydrogen potassium carbonate in 100 ml of boiled distilled water) (*b*) 10 ml of distilled water and finally (*c*) 35 ml of liquefied and somewhat cooled peptone-agar (see above) i.e., enough to pour 3 plates, which can be used immediately and, in contrast to those made with medium 1 retain their selectivity upon storage.

In laboratory tests including those with cholera-contaminated faeces, both media proved most satisfactory while cholera and El Tor vibrios invariably grew well or particularly in the case of medium 2, even abundantly other organisms including *Ps pyocyanea B faecalis alcaligenes E coli* and enterococci failed to grow Unfortunately Vedder & van Dam had no cholera like vibrios at their disposal.

Wahbi (1938) who had the opportunity of using Vedder & van Dam s media for the stool examination of 61 pilgrims at a quarantine station in Iraq stated in a short note that he had isolated El Tor vibrios on plates prepared with these media but not on Dieudonné s medium. Since however some of the previous workers—e g., Neufeld & Woithe (1910) and

(4) Inoculation of faecal specimens of cholera patients or carriers invariably gave a positive result in the case of Dieudonné s and Kabeshima s media, while Pilon s medium failed in some instances.

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Whatever the merits of the substitute media were both before and during the First World War Dieudonné s method was by far the most frequently used in actual laboratory practice by Bürgers (1910) and a considerable number of subsequent workers in Europe as well as by Greig (1913 1917) in India, with equally satisfactory results A large-scale study undertaken during the 1930 cholera outbreak in co-operation with the present writer by the Shanghai Public Health Laboratory (see Pollitzer 1934) also confirmed the value of Dieudonné s method. Still, while parallel tests made with alkaline agar almost invariably gave positive results, absence of growth on the Dieudonné plates inoculated with the same cholera stools or peptone water subcultures, though rare was not excep-

nies, which—in the case of vigorous growth—was surrounded by a zone of clearing Hence though the egg medium was somewhat less selective than that of Dieudonné this untoward feature was counterbalanced by the ease with which the cholera colonies could be picked out The simple method of preparation from readily available ingredients likewise rendered this medium attractive However in a valuable study to which reference has already been made (see page 538) Goldberger (1914) expressed dissatisfaction with the method of Krumwiede and co-workers because he found their medium "to restrain not only the ordinary fecal bacteria but also to markedly inhibit the growth of cholera itself" Goldberger therefore recommended the following modified egg medium for the selective growth of *V cholerae*

"(*a*) *Alkaline-egg solution* First prepare an egg water by beating up a whole egg (or any multiple) with an equal volume of distilled (or good quality of tap) water Then mix one volume of this with an equal volume of a 6 5 per cent solution of *anhydrous* sodium carbonate and steam for half to one hour

"(*b*) *Meat extract glucose agar* This is prepared as follows Meat extract 3 peptone 10 sodium chloride (c.p) 5 glucose 1 agar 30 distilled (or good quality tap) water 1 000 Steam for 3 hours to bring the agar into thorough solution and decant. Distribute in flasks in convenient quantities and sterilize by steaming for an hour and a half Store and use as needed.

" For use, 1 volume of the alkaline-egg solution (*a*) is well mixed with 5 of the hot freshly melted meat extract glucose agar (*b*) and plates poured.

" The plates, if poured in a quiet room free from dust, may be left to set and dry with the covers off for half to three-quarters of an hour when they are ready for seeding. If such a room is not available or if the plates are not for immediate use it is much better to allow the plates to cool and set with the covers on and to get rid of the condensed moisture and dry the plates in the incubator at 37° This is conveniently done by sliding the dish partly over the edge of the cover in an inverted position."

Note Goldberger added that the latter procedure which prevented contamination was also advantageous in the case of Dieudonné s and related media

On Goldberger s medium, which was translucent cholera and some cholera like vibrios grew well, the colonies showing the distinctive features described by Krumwiede and co-workers while the growth of the ordinary faecal bacteria was markedly restrained The plates, if kept at 15° C remained fit for use at least for 10 days, whereas the alkaline-egg solution, if stored at the same temperature, was found to remain serviceable for 83 days

Comparing the medium described above with an alkaline meat infusion agar he had also devised for selective cultivation of *V cholerae* Goldberger reached the conclusion that

" All things considered, the choice for practical work must fall upon the alkaline-egg glucose agar medium. In comparison with Dieudonné s the alkaline-egg glucose agar permits of a more luxuriant growth of the vibrio colony the vibrio colony is more distinctive in appearance it exercises but little if any less restraint for the common fecal bacteria its ingredients are more generally available and, most notably plates may be used at once "

Pilon (1911)—had observed growth of *V El Tor* on the latter medium, it is difficult to believe that the unfavourable results obtained by Wahbi were due to other than accidental causes.

Lefebvre & Gallut (1937) recommended a cholera-selective medium similar to those of Vedder & van Dam which, as summarized in the *Tropical Diseases Bulletin* (1938) was made up as follows

"(1) Prepare ordinary nutrient bouillon of *p*H 8 (2) Add 3 per cent. agar (3) Sterilize 20 min. at 120° C. (4) Filter and add 40 cc. normal soda per litre (5) Remove precipitate by filtration through cotton wool (6) Distribute in large tubes in amounts of 14 cc (7) Sterilize 15 minutes at 115 C. (8) Prepare 10 per cent solution of commercial crystalline haemoglobin in 10 per cent soda 10 distilled water 90 (9) Prepare a buffer solution (*p*H 9.3) containing 8 volumes of sod. chloride 5 650 glycine 7.505 and 2 volumes of decinormal soda. (10) Mix haemoglobin solution and buffer solution in the proportions respectively of 1 to 2.75 and sterilize for 20 minutes at 120° C. (11) Add 6 cc. of buffered haemoglobin to each 14 cc. of agar medium. (12) Slope (13) Use immediately or preferably after drying for 24 hours in the incubator"

Growth of *V cholerae* on this medium was fairly rapid, the organisms sown from a mixture of different bacterial species appearing in the form of large colonies after an incubation of 10-12 hours, thus anticipating the growth of the concomitant organisms

Since their medium kept at least six months, Lefebvre & Gallut (see also Gallut, 1954) recommended speeding up the diagnostic work by issuing the tubes to field workers for direct inoculation of suspect stools at the bedside of sufferers. As the two workers stated with great reason this procedure would be indicated particularly in cholera affected localities distant from laboratories.

2 Egg media

Krumwiede Pratt & Grund (1912) found it possible to prepare solid media for the selective growth of *V cholerae* by substituting egg white or whole eggs for the blood or blood-derivatives used by the workers enumerated above According to Goldberger (1914) the whole-egg medium of Krumwiede and co-workers, which seemed preferable to a similarly prepared egg-white medium, was made up as follows

"Shake thoroughly equal volumes of egg (whole) and water Then mix equal volumes of this egg water and a 12 per cent solution of crystalline sodium carbonate and filter through a thin layer of cotton. Steam the alkaline egg solution for 20 minutes.

"Three volumes of the alkaline egg solution are mixed with 7 volumes of a 3 per cent peptone agar (salt 0 5 peptone 1 agar 3 water 100) and plates are poured. Allow to set and dry with covers off for 20 to 30 minutes. They are then ready for seeding."

Krumwiede Pratt & Grund stated that on this medium, which in contrast to that of Dieudonné was translucent, cholera vibrios (as well as cholera like vibrios) grew in the form of distinctive colonies examined in transmitted light, they had the appearance of being deeply embedded in the agar and had a peculiar hazy look owing to the presence of a halo round the colo

sediment has settled, the supernatant is decanted in 100-ml quantities into 200-250 ml Erlenmeyer flasks. One adds then per 100 ml of the still hot agar 6 ml of a 10 % solution of sodium carbonicum siccum and steams for 10-15 minutes. Afterwards one incorporates (a) 5 ml each of a 20 % saccharose and a 20 % dextrin solution which have been sterilized through steaming for ½ hour, (b) 0.4 ml of a saturated alcoholic solution of diamond fuchsin and (c) 2 ml of a fairly freshly prepared 10 % solution of sodium sulfite, which has been sterilized through short boiling. After the flasks have been kept in a slanting position for the purpose of sedimentation, the contents of each are used for pouring 2 plates which are dried open and in an inverted position for ½ hour at 50° C or correspondingly longer in the incubator. The transparent medium is then ready for use and, if kept in the dark, remains usable for several days.

As described by Aronson, the cholera colonies which became visible on his medium after an incubation of only 10 hours, were at first colourless but after 15-20 hours showed an increase in size and a bright red colour while *E. coli* colonies did not develop within 15-20 hours. Observations he was able to make with one cholera stool fully confirmed these findings: while growth of *E. coli* was inhibited for 24 hours, the colourless cholera colonies developing after an incubation of 10 hours could be easily identified with bacterioscopic and slide-agglutination tests, the characteristic red colour of the growths becoming manifest 17 hours after inoculation of the plates.

The validity of Aronson's claims soon became the subject of considerable debate. The great usefulness of his method was confirmed by the observations of several workers among which those made by Schürmann & Fellmer (1915) and Stern (1915), partly with actual cholera stools, are particularly noteworthy. However, a number of other observers (see the summaries by Baumgarten & Langer Zuckerkandl, 1917; Hesse, 1920; Kolle & Prigge, 1928) were less favourably impressed by the value of Aronson's medium and tried in part to improve it by lowering its alkalinity or using substitute dyes or other ingredients. Particularly noteworthy in this respect is that Taylor & Ahuja (1938) and Read (1939), continuing to make successful use of Aronson's medium in actual cholera work, while otherwise following the original formula of this author reduced the amount of sodium carbonate solution incorporated into the medium by one sixth.

It was afterwards pointed out by Venkatraman (1949) that a precipitation of rosaniline from the alcoholic solution of fuchsin by alkali was apt to render certain batches of Aronson's medium inhibitory for the growth of *V. cholerae* and that the isolated colonies developing after massive implantation of such plates with cholera cultures tended to be rough in character. Bhaskaran (1953), besides confirming these observations through an elaborate investigation, also established that the rough variants developing under these conditions differed markedly from their smooth parent strains in O antigenic structure and were avirulent for mice. However, notwithstanding these shortcomings, Ahuja et al. (1950, 1951) considered Aronson's medium, the use of which had been recommended in the 1947 instructions for cholera work issued by the British Ministry of Health, to be "generally reliable"

As far as the present writer can judge it is certainly regrettable that neither Goldberger s alkaline-egg medium nor Vedder & van Dam s media have been tried out on a worth-while scale in laboratory work with actual cholera stools

3 Dye-containing media

The use of the medium devised for the differential isolation of *S typhosa* by Endo (1904) [1] for cholera-diagnostic work seems to have been recom mended first by Creel (1911) While considering the subcultivation of peptone water-enriched faecal specimens on Dieudonné agar or the egg medium afterwards described by Krumwiede and co-workers instead of on alkaline agar to be an " unnecessary refinement of the technique " he maintained that

" All the advantages that Endo s medium possesses for isolation of *B typhosus* obtain in cholera work if the alkalinity of the medium is increased to double that usually employed, for on this medium vibrio colonies give a very typical, clear amethystine color "

As quoted by Takano and co-authors (1926) Yoshida (1911) noting that cultivation of *V cholerae* on Endo s medium led to the appearance of scarlet-coloured colonies recommended for cholera-diagnostic work a medium as follows

3 / agar base	1000 ml
10 / sodium carbonate	30-40 ml
Saturated alcoholic solution of fuchsin	4 ml
Pure glucose	30-40 g
10 / sodium sulfite solution	25 ml

On this medium cholera and cholera like vibrios developed in the form of scarlet red colonies, while those of the usual faecal organisms were colourless

To judge from a short quotation by Kolle & Prigge (1928) a medium similar to the one just described was recommended for the isolation of *V cholerae* by Mitsutake (1912) while Stokes & Hachtel (1913) once more referred to the possibility of using Endo s original medium for this purpose However related methods of cultivation began to attract much attention only when Aronson (1915) recommended rendering Endo s medium more selective for cholera-diagnostic work by curbing the growth of *E. coli* through (*a*) an increased alkalinity and (*b*) the use of saccharose (and dextrin) as fermentable carbohydrates. The method of preparing Aronson s medium was as follows

One litre of tap water is added to 35 g of agar in a flask. On the following day 10 g of meat extract, 10 g of peptone, and 5 g of NaCl are added, and the mixture is steamed for 4-5 hours. To obviate filtering, the flask is put in a slanting position and, after the

[1] To manufacture Endo s medium, Harris (1925) recommended ( ) the preparation of an agar base w th the following ingredients per litre of distilled water dibasic potassium phosphate (K_2HPO_4) 3.5 g, peptone 10 g, agar 15 g, and lactose 10 g; and (*b*) the addition of 0.25 g of anhydrous sodium sulfite and 3.5 ml of a filtered 10% alcoholic solution of basic fuchsin per 100 ml of the agar base.

Water	1 000 c.c.	Saturated alcoholic solution of fuchsin	5 c.c.
Starch	10 g.	10 sodium sulphite solution	25 c.c.
Peptone	30 g.	10 crystal sodium carbonate solution	30 c.c.
Salt	5 g.		
Agar	30 g.		

"On this medium the colonies of cholera become visible in 12 hours and are bright scarlet in 20 hours the colonies have a diameter of about 2 mm. and are surrounded with a clear zone. The colon bacilli do not thrive very well and the colonies are greyish white, without any clear zone around them. Differentiation between cholera and the other vibrios on this medium is not possible Cane sugar may be substituted for starch and litmus for fuchsin."

As claimed by Lange (1915 1916) a suitable medium for cholera diagnostic work could be prepared by mixing 6 parts of hot highly alkaline agar (containing 40 ml of 10% sodium carbonate solution per litre) with 1 part of 5% rice starch size (*Kleister*) obtained by gluing up the starch with boiling hot water and afterwards autoclaving. In distinction from other organisms, cholera and cholera like vibrios, after an incubation for 14-20 hours on this medium, showed a peculiar growth, characterized by the presence of dew-drop-like colonies surrounded by a distinct halo—features which greatly facilitated the identification of *V cholerae* with slide agglutination tests and subcultivation of the organisms.

Discussing the value of his medium Lange admitted that if used for the direct cultivation of cholera suspect faecal specimens, it was not quite as reliable as other selective media. He asserted, however that in combination with peptone water enrichment " it was definitely superior to the hitherto-known cholera media"

Bötticher (1915) who had an early opportunity of working with Lange's medium, was not in agreement with the claims made by the latter stating that

" Lange's agar does not show the selectivity for cholera vibrios possessed by Dieudonné agar For less experienced workers it may come into consideration as a substitute for Koch [i.e., alkaline] agar Those familiar with cholera diagnosis will hardly be willing to prefer the halo formation to the characteristic appearance of *V cholera* colonies on Koch agar " [Trans.]

Returning to the method suggested by Stokes & Hachtel and used by Ito Gibson (1916) recommended the use of a medium containing per litre of water 30 g agar 10 g each of peptone and starch as well as 1.5 g sodium bicarbonate to which, after fractionate sterilization, a sufficient amount of a sterile aqueous litmus solution was added to produce a blue colour

As stated by Gibson the *V cholerae* colonies becoming visible on this medium after an incubation of 18 hours already showed a faint pink colour whereas those of other organisms, including even the cholera-like vibrios, still exhibited a blue or whitish colour at this stage After an incubation of 36 hours the colonies of the cholera like vibrios also showed a pink tinge but this coloration was less marked than that of the *V cholerae* colonies,

It has to be added that a method of subcultivating cholera suspect colonies on and in a semi-sloped agar medium which contained mannite and Andrade's indicator[1] has been described by Gohar (1947 1948) It would seem however that this procedure ingenious though it was, has been superseded by the more expedient methods now available for the isolation and identification of *V cholerae*

Mention must finally be made of the use for extensive cholera laboratory studies by Husain & Burrows (1956) of a thionin-glycerol agar medium containing 1% peptone, 0 2% glycerol, 1.5% agar and thionin to a concentration of 10 μg per 100 ml (pH 8 0) As the two workers stated, this medium while giving substantially the same plate counts as plain or blood agar "*allowed maximal differentiation of the colonial types prolonged* viability not obtainable with 2 1 agar and a high degree of stability of colonial type on successive subculture" Substitution of acid fuchsin eosinate of methylene blue or malachite green for thionin was found to give "a slightly reduced differential effect"

4 Starch-containing media

Though Eijkman recorded as early as 1901 that cholera and cholera like vibrios, *in contrast to E coli produced a halo round their colonies when* grown on agar plates containing rice or arrowroot starch Gordon (1906) seems to have been the first worker who proposed to take differential-diagnostic advantage of the marked ability of *V cholerae* to decompose starch Using for this purpose a litmus-tinted fluid medium which contained 0 5% starch besides 1% meat extract, 1% peptone, and 0 1% sodium bicarbonate he found that in this the cholera vibrio alone produced a strongly acid reaction within 24 hours whereas (*a*) Finkler & Prior's vibrio produced only a feeble acidity by the third day of incubation, and (*b*) other organisms including *E coli S enteritidis* and *Proteus* failed to acidify the medium.

Stokes & Hachtel (1913) apparently the first to work with similar solid media, stated that they had been unable to distinguish between cholera and *E coli* colonies when growing the organisms on 3% starch litmus agar plates. They found, however that, when inoculated in 0.55% semi solid agar containing 1% starch and litmus, cholera and cholera like vibrios had a characteristic appearance growing in the form of pink colonies, whereas *E. coli* and other organisms like *B faecalis alcaligenes Proteus* and typhoid bacilli formed blue colonies.

As summarized by Takano and co-authors (1926)

"Ito (1914), taking advantage of the fact that the cholera vibrio changes starch into sugar and then produces acid, while colon organisms do not have any such action, devised the following starch medium

[1] The indicator of Andrade (1906) is prepared by dissolving 0 1-0.5 g of acid fuchsin in 16 ml of N sodium hydroxide and 100 ml of water

Water	1 000 c.c.	Saturated alcoholic solution of fuchsin	5 c.c.
Starch	10 g.	10% sodium sulphite solution	25 c.c.
Peptone	30 g.	10% crystal sodium carbonate solution	30 c.c.
Salt	5 g.		
Agar	30 g.		

"On this medium the colonies of cholera become visible in 12 hours and are bright scarlet. In 20 hours the colonies have a diameter of about 2 mm. and are surrounded with a clear zone. The colon bacilli do not thrive very well and the colonies are greyish white without any clear zone around them. Differentiation between cholera and the other vibrios on this medium is not possible. ... Cane sugar may be substituted for starch and litmus for fuchsin."

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Discussing the value of his medium, Lange admitted that, if used for the direct cultivation of cholera-suspect faecal specimens it was not quite as reliable as other selective media. He asserted, however, that in combination with peptone water enrichment "it was definitely superior to the hitherto-known cholera media".

Böttcher (1915), who had an early opportunity of working with Lange's medium, was not in agreement with the claims made by the latter, stating that

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Returning to the method suggested by Stokes & Hachtel and used by Ito, Gibson (1916) recommended the use of a medium containing per litre of water 30 g agar, 10 g each of peptone and starch, as well as 1.5 g sodium bicarbonate to which, after fractionate sterilization, a sufficient amount of a sterile aqueous litmus solution was added to produce a blue colour.

As stated by Gibson, the *V. cholerae* colonies becoming visible on this medium after an incubation of 18 hours already showed a faint pink colour whereas those of other organisms including even the cholera-like vibrios still exhibited a blue or whitish colour at this stage. After an incubation of 36 hours the colonies of the cholera-like vibrios also showed a pink tinge but this coloration was less marked than that of the *V. cholerae* colonies,

which after an incubation of about 24 hours were already seen to be surrounded by a faint pink halo With the exception of some Gram-positive diphtheroids, no other faecal organisms grew in the form of pink colonies.

Considering his findings, Gibson was of the opinion that his medium was suitable for the direct cultivation of broth-emulsified faecal specimens, including even specimens from suspected cholera carriers—a claim which the present writer for one would not be prepared to endorse

Starch-containing media were also recommended for the selective cultivation of *V cholerae* by Kodama (1921, 1922a, 1922b) and Kinbayashi (1933) as well as more recently by Yen (1947) Ch i & Zia (1949) and Dishon (1951)

Yen (1947) noting that the fluid medium he had devised for the same purpose (see above page 538) rarely yielded pure growths of *V cholerae* recommended hastening the isolation of this organism by the use of an analogous solid medium, prepared as follows

" To 40 ml. egg white [are] added 280 ml. distilled water and 20 ml. *N*.NaOH. The mixture is beaten thoroughly gently boiled for 30 minutes in an evaporating basin and cooled to room temperature. 100 ml. of a 10 % aqueous solution of soluble starch, previously warmed to 40° C and thoroughly shaken for 2 minutes, are then added and the volume made up to 1 liter with distilled water To this are added

Peptone	10 gm.	Maltose	1 gm.
Meat extract	3 gm.	Magnesium chloride	1 mg.
Potassium nitrate	3 gm.	Agar agar	20 gm.
Sodium chloride	3 gm.		

" The whole mixture is heated in a water bath for one hour with frequent shaking, then filtered through cotton, and the reaction of the medium adjusted to pH 8 0 The filtrate is then distributed into flasks in 100 ml. lots and autoclaved at 15 lbs. [1 05 kg per cm²] pressure for 15 minutes. Just before pouring into plates, 1 ml. of 1 10 000 dilution of Malachite Green in 95 % ethanol, and 0.5 ml. of 1 100 dilution of Rosolic Acid in 95 % ethanol are added to each 100 ml. of the medium, and thoroughly shaken until the indicators are evenly distributed throughout the mixture. It is then poured into sterile plates to a depth of about 0.3 cm. During inoculation, the surface of the cooled medium should not contain excessive moisture."

Cultivation of cholera vibrios on this medium for 18-24 hours at 37° C gave rise to transparent colonies surrounded by greenish yellow haloes whereas other organisms, including *E coli Proteus* and *B faecalis alcaligenes* failed to produce such zones of clearing and enterococci failed to grow Yen claimed that his medium had " been found to be of great practical use for the primary isolation of *V cholerae* "

Ch i & Zia (1949) finding Yen s media unsatisfactory tried to improve them by the incorporation of potassium tellurite While the fluid medium they thus manufactured has already been dealt with above (see page 543) their method of preparing an analogous solid medium may be described as follows

To a broth made with 5 g meat extract, 10 g peptone, 1 g KNO_3, 1 g $MgCl_2 \cdot 6H_2O$ 8 g NaCl, and 900 ml distilled water one adds 20 g of agar After this mixture has been

dissolved by heating in a double boiler and the water lost by evaporation has been restituted the pH is adjusted to 9.2. Then, after the medium has been cleared by sedimentation and the sediment discarded, 100 ml of a 5% soluble starch solution, which has been sterilized by boiling for 2 minutes, are added After thorough mixing the medium is distributed in 100-ml lots into flasks and these are sterilized at 12 pounds per square inch (0 8 kg per cm²) for 20 minutes. Just before plates are poured from the medium cooled down to 60 C, one adds to each lot (a) 1 ml sterile 0.2% potassium tellurite solution (aqueous) and (b) 1 ml of a 0.5% rosolic acid solution in 90% ethanol.

According to Chu & Zia, growth of *V cholerae* on this medium was manifested after an incubation of 12 24 hours by the appearance of black colonies. These if found to consist of vibrios, were used for slide agglutination tests with cholera immune serum diluted 1/80 Apart from the occasional development of bluish colonies of coliform organisms the medium inhibited the growth of the normal intestinal flora However while finding their solid medium reliable Chu & Zia were able to arrive at a more rapid diagnosis of cholera with the aid of their fluid medium

The solid medium of Dishon (1951) was prepared with a 2% 2.5% agar base containing 0 6% meat extract 0 5% peptone, 2% NaCl and 1% starch After this medium had been autoclaved, the following ingredients were added per 100 ml 1.5 ml of a 10% sodium sulfite solution 4 ml of a 20% sodium carbonate solution 2.5 ml of a 20% saccharose solution 0.2 ml of a saturated alcoholic solution of acid fuchsin, and 1/200 000 each of gentian violet and brilliant green (final pH 8 5-8 7)

Cholera colonies developing on this medium were transparent and slightly pinkish they had a diameter of 2 5 mm and were surrounded by a clear zone which already began to become manifest after an incubation of 6-8 hours. Nevertheless, as has been indicated above (see page 545) Dishon recommended inoculating cholera-suspect stools first into his fluid medium and transferring material from this to plates of the solid medium after an incubation of 6-8 hours After the plates had been kept in the incubator overnight, isolated colonies were picked out for identification tests Examination of the plates was greatly facilitated by the fact that growth of contaminants was inhibited for 48 hours

As far as could be ascertained, Dishon s media as well as those of Chu & Zia have not yet been tried out in laboratory work under actual cholera conditions

5 Casein-containing media

In the course of his classical investigations on bacterial enzymes Eijkman (1901) found that some species including *V cholerae* had the property of producing haloes round their colonies on milk agar plates as well as on agar media to which instead of milk, a mixture of (a) a solution of casein carbonate and (b) calcium chloride had been added There was no doubt therefore that the halo formation on milk agar plates was the result of an action of the bacterial enzymes on the casein and not on the milk fat

Further establishing that the property of halo production on milk or casein agar plates was possessed solely by gelatin-liquefying species, Eijkman concluded that one and the same bacterial enzyme was responsible for both these phenomena. He pointed out that for diagnostic work it would be more convenient to use milk agar instead of gelatin plates in view of the low melting point of the latter and their progressive liquefaction during incubation. It has to be noted, however, that the property of halo production on milk or casein agar plates was possessed also by many cholera-like vibrios as well as by some other organisms, such as *Ps. pyocyanea*, apt to be met with in cholera suspect stools. It was possibly for this reason that no immediate diagnostic advantage seems to have been taken of Eijkman's method of cultivation. However, in later years casein-containing media were again recommended for the cultivation of *V. cholerae* by Boccolari & Olivi (1916), Ko-Ran (1922a, 1922b), Vardon & Datta Roy (1938) and Koch & Kaplan (1952, 1953).

Boccolari & Olivi (1916) found it preferable to use 6 g of trypsinized casein per litre instead of 10 g of peptone for the preparation of Aronson's medium.

The medium recommended by Ko-Ran (1922a, 1922b) was prepared by adding to 100-ml amounts of a 3% agar base, made up with peptone and sodium chloride only (*a*) a mixture of 0.5 g casein or nutrose and of 2 ml of a 10% solution of anhydrous sodium carbonate in 10 ml distilled water and (*b*) 0.1 ml of a saturated solution of fuchsin in ethanol.[1]

On plates poured with this medium cholera vibrios grew in the form of light-red round colonies surrounded by a clear zone.

Papain-casein digest broth and agar media devised by Vardon & Datta Roy (1938) were found to be satisfactory for the growth of various bacteria including *V. cholerae*. However, since these media would be useful for vaccine manufacture and bacteriophage work rather than for diagnostic purposes, it does not seem indicated to deal here with the details of their manufacture, which are clearly set forth in the well-documented publication of these two authors.

As a result of investigations made with the aim of finding a simple medium for the cultivation of cholera vibrios with an increased yield, Koch & Kaplan (1952, 1953) arrived at the following formula:

Peptone 0.5%, casein hydrolysate 0.5%, sodium chloride 0.5%, disodium hydrogen phosphate 0.25%, Bovril 0.15%, Marmite, 0.15%, glycerol 2.2%, and agar 2.5%.

The two workers found that the yield of *V. cholerae* on this medium was more than twelve times that on plain peptone agar and that the organisms grown according to their method possessed satisfactory immunizing properties. It would seem, therefore, that, like the fluid casein hydrolysate medium utilized by Sokhey, Habbu & Bharucha (1950), mention of which has been made in Chapter 4, and a similar semisynthetic medium with a good yield recommended by Uttberg-Olsson & Billaudelle (1956), Koch & Kaplan's medium would be useful principally for the manufacture of cholera vaccine.

6. *Bile-containing media*

As summarized by Takano and co-authors (1926):

" Toyoshima (1914) took advantage of the fact that cholera grows well in ox bile and substituted it for broth in preparing strongly alkaline agar medium. Cholera grows in

[1] The statement made in the review of Ko-Ran's article by Takano and co-authors (1926) that the ingredients enumerated above were added to one litre amounts of 0.3% agar is obviously due to a misprint.

large colonies greyish white in colour with a moist surface Around the colonies are discoloured zones. The growth of other organisms than the vibrio is inhibited "

An attempt made by Maitra & Basu (1924) to utilize the bile salt/lactose medium—which was devised by MacConkey (1905) for the isolation of *S typhosa* and allied organisms—for cholera work as well proved disappointing while cultivation of about 200 cholera stools on plain alkaline agar was invariably successful parallel inoculation of these specimens on MacConkey s medium gave a negative result in 80% However it deserves attention that as Gohar & Makkawi (1947) afterwards claimed the ingredient in MacConkey s medium responsible for this untoward result was the neutral red which if added in concentrations above 1/20 000 was apt to inhibit the growth of *V cholerae* It is certain that as proved by ample experiences in India (see for instance Brahmachari 1927 Pasricha and co-authors, 1932a Ashehov et al 1933a and Panja, 1947) agar media containing 0.5% sodium choleate (commonly but not quite correctly called sodium taurocholate) were fully suitable not only for the subcultivation of *V cholerae* but even for its direct cultivation from cholera-suspect stools and could therefore be used to advantage for practical purposes Similarly direct cultivation of cholera suspect faecal specimens on desoxycholate citrate agar (a medium described in the standard works on laboratory technique) was recommended in the 1947 instructions for stool examination issued by the British Ministry of Health

Panja & Ghosh (1943) who were unable to prepare desoxycholate citrate agar during the Second World War recommended in its place a medium of the following composition

Meat extract	0.5%	Sodium phosphate	0 75
Peptone	0.5%	Ferric citrate	0.30%
Sodium taurocholate	0 85%	Lactose	1.25%
Sodium citrate	0 80%	Agar	2.50%
Sodium thiosulfate	0 85%	Neutral red (0 25% solution)	1.5 ml/100 ml

Compared with other bile salt-containing media including that of MacConkey Panja & Ghosh s agar gave better results not only with artificially contaminated faecal specimens but also with actual cholera stools The plates of the new medium, which was transparent, remained fit for use for about a week. Chatterjee (1953 1956) recommended that in order to obtain particularly reliable diagnostic results plates of this medium be used side by side with plates of MacConkey s medium and of sodium choleate agar for the direct cultivation of cholera suspect stools

7 *Bismuth sulfite agar*

In their important article on bismuth-sulfite media for the isolation of *V cholerae* already referred to earlier in this chapter (see page 540 et seq) Wilson & Reilly (1940) drew attention to the fact that the solid medium

devised by Wilson & Blair (1931) for work with the typhoid paratyphoid group of bacteria if slightly modified, was also eminently useful for cholera diagnostic work The method of preparing such a modified medium was as follows

"Peptone 40 g. NaCl 20 g., agar 80 g., water 4000 c.c., sodium carb sol. (53 g. to 400 c.c. water) 40 c.c. The medium is autoclaved and without being filtered is adjusted to a reaction of pH 8 6.

"To 100 c.c. of this medium melted and cooled to 50° C are added 20 c.c. stock mannitol saccharose sulphite bismuth solution, 2 c.c. phenol red 1/1000 watery solution, and 2 c.c. absolute alcohol Plates are poured and the surface inoculated

"The stock mannitol saccharose bismuth sulphite solution is prepared as follows
(*a*) 100 g. sodium sulphite anhydrous dissolved in 500 c.c. boiling distilled water
(*b*) 30 g. bismuth ammonio-citrate scales dissolved in 250 c.c. of boiling water
(*a*) and (*b*) are mixed and boiled for two minutes, cooled and then added to (*c*) which consists of 50 g. saccharose and 5 g. mannitol dissolved in 250 c.c of water To the mixture are added 15 g. sodium bicarbonate dissolved in 50 c.c. cold water"

As established by Wilson & Reilly their modified medium, while promoting the rapid and profuse growth of *V cholerae* not only suppressed that of *E. coli* and *B lactis aerogenes (Bact aerogenes)* but also proved to be unfavourable for the development of many cholera like vibrios of 25 such strains tested six only grew well while the growth of 19 was scanty or even nil More important still, the medium was also not favourable for *V El Tor* the colonies of which were much smaller than those resulting from the implantation of "epidemic" strains of classical non-haemolytic cholera vibrios.

Describing the growth appearances of the latter Wilson & Reilly stated that

"On the mannitol saccharose sulphite bismuth phenol red alcohol agar plates colonies of the cholera vibrio appeared after one night's incubation and were yellowish brown in colour In the case of some strains after two days the colonies exhibited a dark metallic lustre. In general the characteristic feature was a yellowish brown growth resulting from the action of the acids produced from fermentation of the mannitol and saccharose on the phenol red."

Wilson & Reilly admitted that somewhat similar colonies were formed on their medium by various strains of the genus *Proteus* which, however were unable to form spreading films of growth For this reason and also because it was easy to differentiate cholera vibrios not only with slide agglutination tests but even by mere smear examination, the ability of *Proteus* strains to grow on the bismuth-sulfite medium did not cause difficulties.

On the basis of their findings, Wilson & Reilly felt entitled to postulate that

"Rich growth on our sulphite bismuth agar medium will be another differential test supplementing serological tests and the finding of Taylor (1937) and others that the true cholera vibrio is (*a*) non-haemolytic of washed goat erythrocytes, (*b*) ferments saccharose and mannose but not arabinose, (*c*) gives a positive cholera red and a negative Voges-Proskauer reaction."

A modified bismuth-sulfite medium afterwards recommended for cholera-diagnostic work in India (see Pandit, 1941 Ahuja et al 1951) had the following formula

2.5 agar base (pH 8.8)*	100 ml
20 sodium-sulfite solution	4.8 ml
Liquor bismuthi	0.16 ml
Absolute ethanol	0.2 ml
10 mannose solution	1.0 ml

Pandit (1941) recommended preparing the agar base with papain-digest broth, whereas Ahuja et al. advised the use of a tryptic digest broth (Douglas 1914) for this purpose.

** Liquor bismuthi was prepared according to the formula given above on page °40

Reporting in 1942 to the Scientific Advisory Board of the Indian Research Fund Association on the examination of 233 cholera-suspect stools, the Director of the School of Tropical Medicine in Calcutta stated that direct plating of these specimens gave 58% positive results in the case of Wilson & Reilly's medium as against 59% positives on bile-salt agar plates. Both these results were thus inferior to those obtained with the enrichment method devised by Panja (1942—see above page 543) which gave 81% positive results

However Pandit (1941) as well as Ahuja et al (1950, 1951) found Wilson & Reilly's modified medium particularly useful for the direct plating of cholera-suspect stool specimens. The last mentioned observers, while admitting that Aronson's medium also exerted an inhibitory effect on many organisms other than vibrios stressed that (*a*) certain batches of the latter medium were "poorly supportive of the growth of *V. cholerae*" and (*b*) in contrast to Aronson's medium that of Wilson & Reilly inhibited the growth of many cholera like strains and even hampered that of *V. El Tor*

It has to be noted that Felsenfeld et al. (1951) comparing various solid media by growing on them bacterial mixtures containing, per millilitre 500-1000 cholera vibrios as well as 10 000-15 000 organisms each of *E. coli* *B aerogenes* *Proteus vulgaris* *Pseudomonas aeruginosa* and an enterococcus, arrived at a less favourable estimation of Wilson & Reilly's medium They recorded the results of their comparative tests as follows

Probability of isolation of at least one colony of cholera vibrios when inoculating 0.01 ml vibrio-mixture to each of two identical plates

Medium	*Chemical composition*	*Probability Poisson distribution*
Alkaline agar	Peptone agar pH 7.8-8.0	91.7
Dieudonné	Alkaline blood agar	67.8
Krumwiede	Alkaline egg agar	72.5
Aronson	Alkaline-sucrose-dextrin-agar—Andrade	97.8
Wilson & Reilly	Alkaline bismuth-sulfite agar	85.7
Teague & Travis (1916)	Alkaline eosin-vesuvin agar	81.2
Panja & Ghosh	Bile-salt agar	90.5

As given by Gradwohl (1948) this medium was prepared by adding 1 g of sucrose 2 ml of 3 / aqueous solution of eosin B and 4 ml of a 1/1000 aqueous vesuvin (Bismarck brown) solution per 100 ml of 2 / 3 beef extract agar (pH 8.0).

devised by Wilson & Blair (1931) for work with the typhoid paratyphoid group of bacteria, if slightly modified was also eminently useful for cholera diagnostic work The method of preparing such a modified medium was as follows

"Peptone 40 g., NaCl 20 g., agar 80 g., water 4000 c.c., sodium carb sol. (53 g. to 400 c.c. water) 40 c.c. The medium is autoclaved and without being filtered is adjusted to a reaction of pH 8 6.

"To 100 c.c. of this medium melted and cooled to 50 C are added 20 c.c. stock mannitol saccharose sulphite bismuth solution, 2 c.c. phenol red 1/1000 watery solution, and 2 c.c. absolute alcohol Plates are poured and the surface inoculated.

"The stock mannitol saccharose bismuth sulphite solution is prepared as follows
(a) 100 g. sodium sulphite anhydrous dissolved in 500 c.c. boiling distilled water
(b) 30 g. bismuth ammonio-citrate scales dissolved in 250 c.c. of boiling water
(a) and (b) are mixed and boiled for two minutes, cooled and then added to (c) which consists of 50 g. saccharose and 5 g. mannitol dissolved in 250 c.c. of water To the mixture are added 15 g. sodium bicarbonate dissolved in 50 c.c. cold water"

As established by Wilson & Reilly their modified medium, while promoting the rapid and profuse growth of *V cholerae* not only suppressed that of *E. coli* and *B lactis aerogenes (Bact aerogenes)* but also proved to be unfavourable for the development of many cholera like vibrios of 25 such strains tested six only grew well while the growth of 19 was scanty or even nil More important still, the medium was also not favourable for *V El Tor* the colonies of which were much smaller than those resulting from the implantation of "epidemic" strains of classical non haemolytic cholera vibrios

Describing the growth appearances of the latter Wilson & Reilly stated that

"On the mannitol saccharose sulphite bismuth phenol red alcohol agar plates colonies of the cholera vibrio appeared after one night's incubation and were yellowish brown in colour In the case of some strains after two days the colonies exhibited a dark metallic lustre. In general the characteristic feature was a yellowish brown growth resulting from the action of the acids produced from fermentation of the mannitol and saccharose on the phenol red."

Wilson & Reilly admitted that somewhat similar colonies were formed on their medium by various strains of the genus *Proteus* which, however were unable to form spreading films of growth. For this reason and also because it was easy to differentiate cholera vibrios not only with slide agglutination tests but even by mere smear examination, the ability of *Proteus* strains to grow on the bismuth-sulfite medium did not cause difficulties.

On the basis of their findings Wilson & Reilly felt entitled to postulate that

"Rich growth on our sulphite bismuth agar medium will be another differential test supplementing serological tests and the finding of Taylor (1937) and others that the true cholera vibrio is (a) non-haemolytic of washed goat erythrocytes, (b) ferments saccharose and mannose but not arabinose, (c) gives a positive cholera red and a negative Voges-Proskauer reaction."

duct after dissolution through cambric, sterilizing it and admixing it when needed in a proportion of 3 : 7 to a neutral agar base Like Dieudonné's agar the plates poured from this medium required a ripening period of 24 hours. However while otherwise possessing qualities comparable to those of Dieudonné's medium, the alkaline meat agar plates had the advantage of being transparent

While none of the modified media just described has been permanently adopted, a study of the newer literature as well as of the recent instructions for cholera laboratory work (Seneca & Henderson 1949, Ahuja et al 1950 1951 Gallut 1954) shows that notwithstanding the introduction of special selective media the use of plain alkaline agar plates has been continued This is not surprising if it is considered that under certain conditions the latter media are apt to give as satisfactory results in cholera-diagnostic work as the former This is particularly true if the solid media are used merely for the subcultivation of preliminarily enriched faecal specimens collected from patients in the acute stage of the disease

There can be no doubt, however, that if sole reliance is placed upon the rapid method of directly plating the faecal specimens—a procedure already recommended by a few of the earlier workers, e g. by Rivas & Smith (1912) and increasingly used in recent years—cultivation on highly selective media is indicated However even these give reliable results only with the vibrio-rich faecal specimens collected from patients in the acute stage of cholera, but not with the stools of cholera convalescents or carriers, which usually contain only scanty numbers of the causative organisms It is generally agreed, therefore that preliminary enrichment of the latter kind of specimens in fluid media is indispensable If as is indicated, the highly effective enrichment methods now available are applied for this purpose, it seems a moot point whether it is necessary also to use highly selective solid media for subcultivation

Examination of Vomits

The contention made in Chapter 6 that the occurrence of the causative organisms in the vomits of cholera patients is by no means as exceptional as has been claimed by some authors is well supported by systematic observations made in this respect by Panja Malik & Paul (1942) Examining single specimens of the vomits of 52 cholera patients both with the aid of peptone water enrichment and by direct plating on bile-salt agar these workers were able to isolate *V cholerae* 26 times i.e. in 50% of their material As was to be expected they obtained positive results more frequently if the pH of the vomits was above 6 0 (as was usually the case) whereas at a pH below 5 0 no isolations were made

However valuable as these observations are they render it clear that an examination of the vomits of cholera patients could only supplement but not replace that of faecal specimens

It would not seem wise, however, to lay greater stress upon the observations made under rather artificial as well as rigid conditions by Felsenfeld et al. than upon the favourable results obtained with the modified medium of Wilson & Reilly (not tested by Felsenfeld et al.) in the course of laboratory work with actual cholera stools. As far as the present writer can judge, it is the involved method of preparation and not any lack in reliability which limits the usefulness of this medium.

While, as discussed above many investigators were intent upon devising specially selective media for cholera-diagnostic work some continued to advocate the use of plain alkaline agar for this purpose

Thus Creel (1911), describing the methods used at the New York Quarantine Station for the detection of cholera carriers, stated that, in order to save time and labour plain alkaline agar media were used for making the subcultures from the originally inoculated peptone water cultures.

Similarly Babes (1914 see also Neumann, 1915) recorded that when having to make mass examinations of cholera-suspect stools during the Balkan wars, he resorted to subcultivation on plain alkaline agar He first used agar tubes for this purpose, starting inoculation at the bottom of the slants and continuing it upwards in a zigzag course He claimed that by following this technique it was possible to find isolated colonies of *V cholerae* at the top of the slants after incubation for only six hours. Later in order to save glassware and labour Babes distributed layers of plain alkaline agar on the inside of 1-litre bottles and used these for the inoculation of 20 specimens in the manner just described.

Volpino (1916) considered subcultivation of peptone-water-enriched faecal specimens on plain alkaline agar as satisfactory as that on Aronson s medium, on which the characteristic appearance of the cholera colonies was apt to become manifest with some delay

Attention has to be drawn also to various procedures devised by some of the earlier workers to increase the selectivity of plain agar media for *V cholerae*

Crendiropoulo & Panayotatou (1910) tried to reach this goal by separately preparing (1) a 3 % agar base containing 1 % peptone and 0.5 % sodium chloride and (2) an alkaline peptone solution made by (*a*) dissolving 5 g of peptone in 190 ml of tap water· (*b*) adding, according to the kind of peptone used, 8-10 ml of a 10 % solution of caustic soda (*c*) after short heating and subsequent cooling of this mixture, filtering it through paper and, finally (*d*) sterilizing it for 1 hour in the steam-sterilizer

Immediately before use 4 parts of this alkaline peptone solution were admixed under aseptic conditions to 6 parts of the agar base, and plates were poured.

Crendiropoulo & Panayotatou claimed that their new medium, besides being easily preparable and transparent, had the advantage of inhibiting the growth of the usual faecal bacteria and—in contrast to Dieudonné s agar—considerably retarding that of *Ps pyocyanea* However while Crendiropoulo (1912) recorded satisfactory experiences with the new medium in actual cholera work, Goldberger (1913) found it insufficiently selective.

Tokunaga (1911) recommended, for the isolation of *V cholerae* the use of a serum-containing alkaline agar which, being transparent—in contrast to Dieudonné s medium—facilitated diagnostic work. For the same reason, Violle (1915) advocated the use of an alkaline agar medium to which 10 % of glycerol had been added.

The alkaline meat-agar (*Fleischnatronagar*) devised by Esch (1915) was prepared by heating 500 g of meat (or of fish) in 250 ml of normal soda solution, filtering the pro-

"To furnish final and exact proof that the water was not unjustly incriminated namely the discovery of the cholera bacteria in the suspected rivers was not possible Competent and most experienced observers examined the Elbe water at Hamburg and the Spree water at Berlin most painstakingly yet no positive findings rewarded their endeavours" [Trans.]

But, Fraenkel aptly continued

"For the expert this is not surprising. For in view of the extraordinarily small quantities to which bacteriological water analysis is necessarily restricted, and more still on account of the abundance of various saprophytes in surface water it must be considered a lucky accident of the first order if one nevertheless succeeds in getting hold of the cholera bacteria." [Trans.]

It is not at all surprising that under these circumstances the thought arose of improving the results of water examination by resorting in place of direct platings with a few drops of the samples, to the enrichment of larger quantities of the suspected waters In fact as will be discussed soon such an improved technique was suggested as early as 1892 by Heim However before dealing with this proposal it seems well first to refer for the convenience of the record to an alternative method devised by Arens (1893) for the discovery of small numbers of cholera vibrios in water samples

For this purpose Arens advised the use of 175-ml quantities of the suspect waters to which 25 ml of a broth, prepared according to the method of Karliński (1890) from cattle pancreas, and—further to promote the growth of *V cholerae*—1 ml of a 10% caustic potash solution were added. As Arens established through laboratory tests, it became possible in this way regularly to isolate cholera vibrios by subsequent plating from specimens containing not more than two of the organisms per 5 ml of water Occasionally positive results were obtained with samples containing one cholera vibrio per 5 ml—once even with a specimen containing one organism per 30 ml.

While in spite of its apparent efficacy Arens method seems never to have been used in actual practice the procedure originally suggested by Heim (1892) soon attracted universal attention As this worker summarized in 1901 he had pointed out in his original article

that the detection of cholera vibrios in water is rendered easier if one takes instead of the usual amount of 1 c.c. a larger quantity of the suspected water and prepares through the addition of substances suitable as pabulum for the bacteria a substrate in which the vibrios, on account of their oxygen requirement, come to the surface and, assembling there, form a membrane from which they can easily be isolated. Having examined several such nutritive substances, I particularly recommended peptone and sodium chloride" [Trans.]

For work with actual specimens Heim (1892) advised adding to at least 250-500 ml of the waters to be examined sufficient amounts of these two substances to obtain a peptone concentration of 1% 2% and a NaCl content of 0 5%. The incubated specimens were examined daily by direct platings on gelatin and by broth subcultures, both made with material from the surface membranes of the growths

The value of the method just described was endorsed by Flügge (1893) as well as by Koch (1893) and accordingly the following procedure for the

A unique observation by Lieou (1938) deserving attention at the present juncture concerned an individual who succumbed within less than 24 hours to an illness characterized solely by severe gastric pains, anuria, and collapse. Since food poisoning was suspected, the stomach contents obtained at autopsy were used for subcutaneous inoculation of a guinea pig and a rabbit as well as for intraperitoneal injection of a mouse. The first mentioned animal, which had received a dose of 2 ml succumbed after about 30 hours, showing at autopsy a sero-purulent infiltration at the site of injection and congestion of the abdominal organs. Cultivation of its heart blood led to the isolation of *V. cholerae*. There can be no doubt that an adequate bacteriological examination of the stomach or intestinal contents of this victim (which was omitted for the not very cogent reason that the material had not been collected under sterile conditions) would have led to an identical result. In fact, material for such tests might have been obtained during the life of the sufferer either through rectal swabbing or with the aid of an enema, or—as was afterwards suggested by Panja, Malik & Paul (1942)—by giving the patient sterile water to drink so as to induce vomiting.

Water Examination

The history of the examination of water samples for the presence of *V. cholerae* goes back to Koch who stated at the 1884 cholera conference in Berlin that by cultivating small quantities of such specimens directly on gelatin plates he had

"succeeded in finding comma bacilli with all their characteristic properties in a tank which supplied the drinking and otherwise used water for all people living in the vicinity and in the immediate environment of which a number of fatal cholera cases had occurred." [Trans.]

As can be gathered from the literature, especially from a valuable summary by Prausnitz (1903), some other early workers, using the same technique as Koch, also claimed to have isolated cholera vibrios from surface-waters, or from wells or other sources, e.g. the bilge-water of a steam-tug coming from the infected port of Hamburg (Lubarsch, 1892). However, as maintained by some observers, such as Gruber (1894) and Prausnitz (1903), none of these early isolated strains could be properly identified so that, as the latter author put it, "the question of their cholera nature must remain open."

However, even if these early claims as to the occurrence of cholera vibrios in surface or other waters could have been accepted at face value, the scantiness of such allegedly positive findings was certainly disproportionate to the apparently paramount role played by contaminated surface waters in the contemporaneous cholera outbreaks. Stressing this discrepancy, Fraenkel (1892) thus wrote in a little noted but interesting article

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examination of cholera-suspect water samples was prescribed in the regulations for cholera laboratory work compiled by Koch Kirchner & Kolle (1902)

"To 1 litre of the water to be examined one adds one flask [100 ml] of the peptone stock solution [1] and shakes thoroughly the mixture is then distributed in 100-ml quantities into flasks and, after an incubation of 8 and 18 hours respectively at 37° C, these are examined by (*a*) using drops removed from the surface for microscopic examination, and (*b*) making from the flask which shows most vibrios peptone water subcultures as well as inoculating gelatin and agar plates, which are then handled like those made from stool specimens. The pure cultures isolated are identified with the aid of agglutination and Pfeiffer's test." [Trans.]

A similar technique was still recommended in the 1916 German instructions for cholera laboratory work (see Kolle & Prigge 1928) with the difference that, instead of subcultivation in peptone water tubes and on gelatin and agar plates, direct inoculation on Dieudonné and agar plates was prescribed. Similarly Gibson (1916) recommended direct cultivation of the peptone water-enriched water samples on his starch agar medium (see above, page 561) However in the course of extensive studies made from 1932 to 1936 on the occurrence of cholera and cholera like vibrios in the Shanghai surface waters, which were as a rule highly contaminated, the present writer found it more adequate to resort before plating to subcultivation of the enriched water samples in peptone water tubes Plating on plain alkaline agar then gave satisfactory results. He established on the other hand that it was sufficient to collect one specimen of each water to be examined in a sterilized, 300-ml, flat, medicinal bottle and to incubate this directly after a corresponding amount of peptone stock solution had been added

It was gratifying to note that Taylor & Ahuja (1938) in order to study the water vibrios of North India, had independently adopted an analogous, though more refined, technique

Taylor & Ahuja collected 200-ml amounts of the waters to be examined in 10-ounce (about 280 ml), screw-capped, flat, medicinal bottles and then added 20 ml of a stock solution containing 10 % peptone and 5 % NaCl. Alternatively for the collection of water samples in distant localities, bottles containing this stock solution were issued and 200 ml of the water samples to be examined were added in the field. In both cases the reaction of the samples was raised to pH 9 0 by the addition of N/1 NaOH thymol blue serving as indicator

After incubation overnight (done at room temperature during the hot season) 2 ml amounts of the enriched specimens were added to 10-ml quantities of peptone water and then, after an incubation for 6 hours, 1 drop of the fluids was used for the inoculation of Aronson plates containing only 5/6ths of the originally recommended amount of sodium carbonate

A new chapter in the history of the subject presently under review may be said to have commenced in 1939 when Read proposed to replace the

[1] The peptone stock solution was prepared by (*a*) dissolving by heat in 1 litre of sterile distilled water 100 g peptone, 100 g sodium chloride, 1 g potassium nitrate, and 2 g crystalline sodium carbonate; (*b*) after filtration, distributing the solution in 100-ml quantities into flasks and (*c*) autoclaving these.

hitherto practised enrichment method of water samples to be examined for the presence of *V cholerae* by filtration of large amounts of the waters the vibrio-rich residue collected on the filters then being used for cultivation

Read resorted to filtration either through Seitz filters or through kieselguhr impregnated filter papers. In regard to the first procedure he stated

> Using the bismuth-sulphite original modification and passing one litre of water through a Seitz filter and inoculating the disc better results were obtained especially when 2 per cent NaCl were added to the water and the reaction was raised to [pH] 9.2 immediately after inoculation
>
> "The samples were run through the Seitz filters by gravity through the pressure type filters using about six feet [2 m] head of pressure"

To carry out the second method,

> "Three hundred c.c. of 0.5 / Kieselguhr [porous diatomite] were run through a 6-inch [15-cm] filter-paper The water was held in large 4-litre flasks closed with a rubber cork containing a moderate-sized glass tube These were inverted in the funnel in such a manner that the outer end of the tube just reached below the surface of the fluid in the funnel. Filtration then proceeded automatically until the flask was emptied. About 15 litres could be passed through two filter-papers in 5 to 6 hours. The filter-papers were then folded and placed in 60 c.c. or so of bismuth-sulphite enrichment medium and incubated overnight."

Actually using the filtration method in field tests, to which reference has already been made on page 541 Seal (1939) adopted the following technique

> "Samples of water were collected in sterile quart [litre] whisky bottles by dipping them directly into the source To each of these bottles two teaspoonfuls of common salt were added to make the concentration between 1 and 2 per cent, each bottle accommodating about 750 c.c. of water if filled to the brim. For purposes of comparison water samples were also collected in 250-c.c. screw-capped medical bottles containing 20 c.c. of 10 per cent peptone and 10 per cent NaCl. This medium was directly inoculated with water at its source the total volume being made up to 200 c.c Eight drops of N/1 NaOH were then added to increase the alkalinity of the specimen."

Continuing the description of his technique Seal stated that

> The salted samples were passed through Seitz filters fitted with 6-cm discs in quantities varying from 300 c.c. to 1 000 c.c. The discs were inoculated each into 20 c.c. of the selected medium freshly prepared in 100-c.c. wide-mouthed glass-stoppered bottles. The pH was adjusted to 9.2 and the bottles [were] incubated overnight at 37°C. Subsequent treatment was the same as in stool culture"

Summarizing his findings Seal stated that whereas with the aid of peptone water enrichment only 5 out of 117 tank water specimens proved positive for *V cholerae* the number of successful isolations obtained with filtration and subsequent enrichment in the bismuth-sulfite medium (modification of Read, 1939) rose to 8

Read & Pandit (1941) studying the distribution of the cholera and El Tor types of vibrio in rural areas of India, resorted to the kieselguhr method

instead of Seitz filtration of their water samples. Describing the handling of these specimens, the two workers stated that

"1,500 c.c. of water from each source were collected in sterilized whisky bottles. To 1,000 c.c. was added by means of a scoop, common bazaar salt so that the salt concentration was about 1 per cent and sufficient alkali to raise the pH to 9.2. This was then filtered through Kieselguhr impregnated filter-papers the water being poured through funnels by hand. In the Bihar and Sind investigations an improved method consisting of the use of funnel covers and automatic levelling apparatus similar to that described for 'phage filtration by Pandit (1934) was used. After filtration, the filter-papers were folded in small packets with due regard to asepsis and covered consecutively with (*a*) cellophane, (*b*) vaseline paper (*c*) cellophane and (*d*) ordinary brown paper. The packet was then ready for despatch to the central laboratory for inoculation into the bismuth sulphite medium."

A study of the results recorded by Read & Pandit, to which due attention will be paid in a later chapter certainly shows the outstanding value of their method of water examination.

Kieselguhr impregnated filters were also used by Venkatraman, Krishnaswami & Ramakrishnan (1941) in order to study the occurrence of El Tor vibrios in natural sources of water in the absence of cholera. Details of the technique applied for this purpose were as follows

"2,500 c.c. quantities of water were collected from each source, sufficient sea salt added to make a 1 per cent concentration and the pH brought up to 9.2 by the addition of sufficient N/1 NaOH solution, at the spot of collection. These were then transported to the Laboratory generally within 3 to 4 hours (often within one hour) and filtered through Kieselguhr impregnated filter-paper which, with the deposit, was then taken in 100-c.c. stoppered bottles containing 60 c.c. of the mannose-bismuth-sulphite medium and incubated overnight. Platings were made on Aronson and agar media from the enriched cultures."

Examining a total of 878 samples of water collected from 237 different sources, Venkatraman and co-workers were able to isolate El Tor vibrios with the above method in 15 instances and classical non haemolytic vibrios two times.

Panja & Ghosh (1947) in order to utilize the method of isolating *V. cholerae* devised by Panja (1942—see above page 543) for stool examination for the examination of water samples as well, adopted the following technique

"Three to five c.c. of unconcentrated river water from each sample were put into the porcelain candle and incubated for one or two days. A few drops of the surrounding boric-peptone water were then placed on bile-salt agar."

The two workers stated that, examining in this way 524 samples of water collected from the Hooghly River at Calcutta, they had isolated cholera vibrios on 16 occasions. They added that they had compared the efficacy of their method with that "of filtration through filter paper adopted by Dr S. R. Pandit" (? the kieselguhr method) and claimed that the latter had given less satisfactory results. However to judge from their protocols this difference was marked only as far as the isolation of *V. cholerae* in pure

culture was concerned As was to be expected the candle method gave incomparably better results than direct plating of the water samples on bile salt agar

As has been stated already when dealing above (page 543) with the original recommendation of Panja (1942) there can be no doubt that the method devised by this worker is efficacious and this was again confirmed by the experiences with water samples just recorded However as has been pointed out by the present writer when commenting upon Panja s observations practical difficulties militate against the large scale use of his method when handling cholera suspect stools. The same objection holds true if more than occasional water samples have to be tested for the presence of *V cholerae*

It deserves attention that Gohar & Makkawi (1947) claimed to have obtained good results when enriching such water samples with peptone stock solutions containing potassium tellurite However their findings made solely with artificially cholera-contaminated specimens need confirmation through field trials The same holds true of the attempt made by Felsenfeld & Rokkaku (1956) to adapt the method of membrane filtration now amply used for water examination in general to the recovery of cholera vibrios from water samples Describing the technique they used for tests with specimens to which *V cholerae* alone or also *E. coli* and *Aerobacter aerogenes* had been added the two workers stated that

" Millipore filter (MF) disks were used in conjunction with pyrex millipore filter holders. The holders were sterilized in boiling water for 2 min between operations. After the filtration of artificially contaminated water the filter disks were put on the surface of modified Aronson plates in individual petri dishes. This medium consisted of beef extract, 0.3 per cent pancreatic digest of casein (Difco) 1.5 per cent agar 1 per cent sucrose and dextrin, 1 per cent each sodium carbonate 0.5 per cent basic fuchsin, 0 012 per cent and sodium sulfite, 0.2 per cent. The disks were observed under the dissecting microscope and with the naked eye after incubation for 6 and 24 hr All tests were carried out in duplicate."

As shown by comparative tests with samples enriched with peptone water and afterwards plated the membrane filter technique proved superior as well as expedient. One must fear however that in the countries where cholera is frequent the use of this new method will be difficult for financial reasons. Nevertheless, it would be important to compare its efficacy under field conditions with that of the kieselguhr and potassium tellurite methods

Identification Tests

Introductory remarks

Ever since the discovery of *V cholerae* it has been generally agreed that the demonstration of this organism in the stools of the patients is not merely the best, but practically the only means of differentiating between true cholera and other morbid conditions in which merely signs of a choleraic

affection were present. However for more than a decade after the causative organisms of cholera had been found, no permanent agreement could be reached regarding the choice of the methods suitable for their identification It was soon realized that the problem at issue was not simply that of demon strating the presence of vibrios with the aid of the bacterioscopic and culture methods devised by Koch but that the main stress had to be laid upon a differentiation of the cholera from the cholera like vibrios detected by successive workers in ever increasing numbers In spite of the assertions of some of the early observers, it was soon shown that none of Koch s original methods sufficed for the latter purpose As will be further discussed below the hope that the cholera red reaction recommended in 1886-87 would fill this gap was likewise soon dispelled. For reasons which have been set forth partly in the sixth chapter and will be further referred to below experiments with various laboratory animals also failed to give results permitting an invariably clear-cut differentiation between cholera and cholera like vibrios.

Firm ground was thus reached only after the introduction of serological methods for cholera laboratory work by Pfeiffer (1895) and Gruber & Durham (1896) Though the reliability of Pfeiffer s test was far greater than that of the early agglutination methods and although it has retained its outstanding value in spite of improvements in the latter and the introduction of alternative serological procedures, practical difficulties militated from the first against its use in routine cholera laboratory work. It is not surprising therefore, that Pfeiffer s method fell into disuse even before there was valid justification for abandoning it. This is well illustrated by the fact that this test, which had been still referred to in detail in the 1907 German instructions for cholera laboratory work quoted by Kolle & Schür mann (1912) was no longer recommended in the 1916 revision of these instructions (see Kolle & Prigge, 1928) Since, however other reliable methods, particularly improved agglutination tests are now available for practical cholera laboratory work there really does not seem to be any indication for continuing the use of Pfeiffer s reaction for routine purposes. It appears to be unnecessary therefore at the present juncture, to deal once more with this method, the principles of which have already been discussed in the fourth chapter of this book.

As will be gathered from the discussion in Chapter 4 a number of alter native serological methods have successively been recommended for use in cholera laboratory work side by side with, or even in place of the agglutina tion tests In view of the fact, however that none of these substitute procedures surpasses the agglutination method in practical value and that they are almost invariably less expedient, it also seems unnecessary to deal further with them in the course of the present disquisition. On the other hand it is important to pay additional attention to the problem of haemolysis tests, which are indispensable for a differentiation of the true cholera vibrios from

El Tor strains in the strict sense The attempts made by some workers to use bacteriophage tests for diagnostic purposes also require consideration The cholera red test and the problem of animal experimentation will be dealt with together with other methods of confirmatory value in the concluding section of the present chapter

Agglutination tests

Inasmuch as the subject of agglutination received full general consideration when the problems of cholera immunology were discussed in Chapter 4 all that needs to be done now is to deal with (*a*) the methods of manufacturing agglutinating sera (*b*) the technique of performing the agglutination tests and (*c*) the problem of making such tests with rough or otherwise dissociated cholera vibrios In considering these points attention will be paid solely to the technique of O-agglutination because as has been proved by ample recent experiences this alone gives diagnostically valid results in cholera laboratory work.

(*a*) *Manufacture of O-agglutinating sera* Theoretically the necessity of separately preparing O agglutinating sera for the purposes of cholera diagnosis could be obviated by observing the action of the formerly used H+O sera, prepared with living or formolized vibrios on boiled suspensions of the organisms under test In actual practice however this alternative procedure would be not only inexpedient but also rather undesirable because as was established through the pioneer observations of Gardner & Venkatraman (1935) the boiling of the organisms about to be tested is apt to decrease their O-agglutinability Accordingly the principle of working with specific O agglutinating sera, manufactured with boiled suspensions of suitable *V cholerae* strains (or otherwise prepared O antigens) has been generally adopted

It also seems to be the consensus of opinion that—in place of the larger animals (horses or preferably donkeys) that were sometimes formerly used—rabbits ought to serve for the manufacture of O-agglutinating sera.[1] However no full agreement has been reached regarding the details of preparing the O antigens, the route of their administration or the size and number of the antigen doses it is advisable to utilize in order to obtain sera with a satisfactory titre The following rather divergent recommendations made in these respects deserve attention

Describing their techniques, Gardner & Venkatraman (1935) stated that

" Pure O sera were made with saline suspensions from agar [cultures] boiled for 2 hours. Two doses of 0.5 and 1 0 c.c. at a week s interval generally gave sera of 1000-2000 O titre and no method of dosage was discovered that would consistently improve on this, though three doses of a fivefold denser suspension appeared sometimes to be a better stimulus."

[1] Still, the value of immune sera raised in horses for cholera laboratory work has been upheld by a few modern workers such as Iida (1953) and Azarginova and colleagues (1956).

Gardner & White (1937) briefly referred to the manufacture of O-agglutinating sera by various institutes in India with a dry antigen prepared by White through alcohol extraction of cholera vibrios followed by steaming and washing with ether (see White, 1948) They noted that in Kasauli 4 doses of this O antigen were administered to rabbits at 4-5 days interval, starting with a dose of not more than 0.25 mg. At Shillong a longer course of immunization was resorted to an initial dose of only 0 1 mg being followed at weekly intervals by gradually increased doses up to 1 mg or if no satisfactory titre had been attained in this manner even up to 1.5 mg. However Gardner & White, probably influenced by the experience of Gardner & Venkatraman to the effect that such a prolonged course of immunization was of questionable value, were in favour of the method adopted at Kasauli.

Gallut & Grabar (1943) prepared O-agglutinating sera with alcohol-killed antigens, giving during a period of 20 days 5 injections increasing from 0.25 mg to 5 mg of the dry weight of the organisms, and bleeding the animals 7 days after the last injection It has to be noted in this connexion that Gardner & Venkatraman strongly objected to the method of using alcohol-killed antigens for the manufacture of O-agglutinating sera. How ever as will be recorded below Gallut & Grabar s procedure has been used again with apparent success by Gallut (1949)

The simple and apparently satisfactory technique adopted by Tang, Chu & Wong (1944) for the manufacture of O-agglutinating sera was (*a*) to wash off *V cholerae* growths on agar with normal saline and to boil the resulting suspensions for 2 hours in the water bath (*b*) to dilute the sus pensions to a standard of 10 milliards of organisms per ml (*c*) to inoculate rabbits first at one day s interval with 0.5 and 1 0 ml amounts of the sus pensions, respectively by the subcutaneous route and then to administer after a rest period of 4 days, the same doses at one day s interval intra venously and (*d*) to bleed the animals on the 5th day after the last injection.

In the course of their studies on the antigenic structure of cholera and related vibrios Burrows et al. (1946) found that the method of boiling the vibrio suspensions for two hours according to Gardner & Venkatraman s method " was not sufficient to destroy all effective trace of H antigen but that boiling the suspension for 2 to 3 hours under a reflux condenser gave an antigen which did not stimulate the formation of agglutinins to the H antigen "

In order to prepare O sera, Burrows et al resorted to a process of hyper immunization adapted to the reactions of the individual animals. They found that

" The best immune response was given by young animals weighing about 2.5 to 3 kg and subjected to a course of inoculation sufficiently rigorous to prevent gains of more than 100 g in body weight per week. This ordinarily consisted of a series of 5 inoculations at 3-4 day intervals, the first 2 intraperitoneal and followed by 3 intravenous inoculations.

The first inoculation was 1 ml of a suspension containing 10-20 thousand million vibrios per ml and the dose doubled with succeeding inoculations except that in the transition from the intraperitoneal to the intravenous route the dose was not increased and when loss in weight occurred the dose was not increased. Occasional animals did not tolerate the rapid acceleration and gave an inferior immune response In most instances however the immunization was tolerated with maintenance or slight gain in body weight, and peak titer was reached after the third intravenous inoculation as indicated by trial bleeding. If the titer at this time was less than 1 : 20 000 2 more intravenous inoculations were given without increase in dosage and the animal bled out 3 or 4 days after the last inoculation."

When trying to prepare O-agglutinating sera Gohar & Makkawi (1948a) used one of the following two methods to reduce the high mortality of the rabbits undergoing immunization they either injected their animals repeatedly at short intervals intramuscularly, completing the course of immunization with the aid of one intraperitonal and one intravenous injection of boiled *V cholerae* suspensions or resorted to intravenous injections of sensitized suspensions prepared thus

"To a thick suspension boiled for two hours, one-tenth its volume of a high-titre O-serum was added the mixture was then incubated at 37°C. for two hours and centrifuged and finally the deposit [was] washed with saline and resuspended in saline to the required density "

Three injections of this sensitized antigen resulted in the production of sera with the quite satisfactory titre of 1 1250 with practically no mortality among the rabbits undergoing immunization

For his studies on cholera immunology to which reference has been made in Chapter 4 Gallut (1949) worked with antigen suspensions prepared according to the method of Burrows et al (see above) containing 10 milliards of cholera vibrios per ml He used rabbits of an average weight of 3 kg to which a series of 5-7 inoculations gradually increased from 1 to 6 ml was given at 4-day intervals, the first two being administered intraperitoneally the following intravenously Since particularly if recently isolated strains were used, the mortality among the animals undergoing immunization was high, Gallut resorted concurrently to the inoculation procedure of Gallut & Grabar (1943) administering in this manner a total of 20 milliards of cholera vibrios Most of the O-agglutinating sera produced by Gallut had a titre of not less than 1 20 000

Kauffmann (1950) in the course of an investigation also referred to in the fourth chapter used for the preparation of O-agglutinating sera a method analogous to that of Bruce White (1948—see also Gardner & White 1937 quoted above) the details of which were as follows

"A 20-hour agar culture was suspended in saline and boiled for 2½ hours in flowing steam. After centrifuging, the sediment was incubated with 96% alcohol at 37° for 4 hours, then centrifuged again and washed twice with acetone. Then the sediment was collected together with a few ml acetone and kept overnight at 37° Of the powder obtained in this way a small portion was ground in [a] mortar stirred with saline and

then injected intravenously into the rabbits in increasing doses. 4 injections were given at intervals of 4-5 days. Ten days after the last injection the animals were bled totally"

As Kauffmann added the O titre of the sera he was able to produce varied between 1 640 to 1 2560 with an average of 1 1280

Singh & Ahuja (1950) briefly referring to the technique of manufacturing O-agglutinating sera in a study devoted to a re-evaluation of the findings recorded by Burrows et al (1946) and by Gallut (1949) stated that they prepared the O antigens necessary for this purpose by keeping the washings of agar cultures of *V cholerae* enclosed in sealed glass ampoules for 2 hours in boiling water containing salt so as to raise the ambient temperature to 101 C. In a further note on the serological analysis of *V cholerae* (see Chapter 4 page 246) Ahuja (1951) insisted with great reason upon the indispensability of using exclusively strains reliably tested for the absence of roughness for the manufacture of O-agglutinating sera for cholera diagnostic work.

Reporting in 1953 on serological studies of the antigens of *V cholerae* Venkatraman referred to the possibility of preparing suitable O antigens by keeping the saline washings of 24-hour agar cultures for two hours at 100 C in a steam-sterilizer Burrows & Pollitzer (1958) in a carefully compiled statement on the laboratory diagnosis of cholera (see also the annex to this book page 991) considered autoclaving of the *V cholerae* suspensions at a steam pressure of 1 2 pounds per square inch (0 07-0 14 kg per cm²) an effective and probably the simplest method of O-antigen preparation The antigen thus obtained was standardized to contain 1 mg per ml dry weight of vibrios (2000 million per ml) and was then used for the immunization of young rabbits, first by the intraperitoneal route and afterwards by the intravenous route according to the method of Burrows and co-workers (1946) described above.

In the course of their pioneer studies Gardner & Venkatraman (1935) were able to confirm the claim originally made by Japanese workers as to the occurrence of *V cholerae* in three serological types. Making absorption tests with sera raised against Inaba and Ogawa strains respectively the two observers found that

" from each type serum the heterologous type vibrio removes all the subgroup agglutinin for itself but leaves a large residue of agglutinins for the other types "

However while these results testified to the presence of a different subsidiary O antigen in the original (Inaba) and variant (Ogawa) types of *V cholerae* and to the possibility of producing type specific sera with the aid of the absorption method numerous observations by Gardner & Venkatraman left no room for doubt that these two types as well as the intermediary (Hikojima) type fell into the cholera subgroup I of vibrios, since (*a*) they were agglutinated by O sera of that subgroup if not to full titre at least to a large fraction of the titre and (*b*) O sera raised against

representatives of the three types similarly agglutinated unheated suspensions of various strains belonging to cholera subgroup I of the vibrios In view of these findings Gardner & Venkatraman recommended that for practical laboratory work a "standard subgroup I O serum" should be manufactured which contained both the main and the subsidiary antigens of that subgroup

Analysing field experiences made in India with O sera which had been manufactured with a dry antigen furnished by White (see above), Gardner & White (1937) also came to the conclusion that

"for the purpose of routine diagnosis it is recommendable to use an O serum of the bivalent or mixed type, while the use of separate [i.e., type-specific] O sera ought to be restricted to scientific or epidemiological observations" [Trans.]

In agreement with this advice Tang Chu & Wong (1944) relied for the purpose of determining the incidence of the serological types of *V cholerae* during the 1942 cholera outbreak at Kunming, China, upon the use of type-specific Inaba and Ogawa sera the preparation of which they described as follows

"For preparation of specific type sera, the Inaba ('original') serum was absorbed with the Ogawa ('variant') suspension and vice versa The absorption test was done by diluting the serum ten times with saline and mixing the diluted serum with 1/10 of its volume of packed bacterial cells which had previously been boiled for two hours. The mixture was then incubated at 37°C. in a water-bath for four hours and placed in the refrigerator overnight. Next morning the mixture was centrifuged for one hour at high speed and the supernatant removed and used as the specific O serum after having the titre for the homologous and the heterologous strains controlled."

However while using this elaborate method of preparing type specific sera and tube agglutination tests for the purposes of their scientific investigations, Tang and co-workers resorted for routine purposes to the preliminary identification of cholera suspect strains through rapid slide tests made with an unabsorbed serum raised against a stock strain of *V cholerae*

As recorded by Ahuja (1951) it remained

"in most laboratories in India the routine procedure for identification and typing of vibrios to test them against sub-group I O anti-sera designated cholera non differential O sera Such anti-sera contain cholera group-specific agglutinins and a proportion of Inaba and Ogawa type specific agglutinins depending on the antigen employed in raising these sera. If the organism reacts against this serum further tests with mono-specific Inaba or Ogawa serum are done to find out their appropriate types."

Similarly as has already been stated in Chapter 4 Kauffmann (1950) recommended using for cholera-diagnostic purposes a polyvalent O serum produced by simultaneous immunization of rabbits with both Inaba and Ogawa antigens and resorting for differential diagnosis between the two types to a serum obtained by absorption of a polyvalent or an Ogawa serum by an Inaba strain

As has been discussed in the fourth chapter these proposals were not in agreement with the conclusions reached by Burrows et al. (1946) and by Gallut (1949) who considering their A antigen the group-specific antigen of the cholera subgroup advocated that exclusive use be made for the serological identification of *V cholerae* of monospecific anti A sera. Since however as further discussed in Chapter 4 the validity of these recommendations has been vigorously opposed by Kauffmann (1950) Ahuja & Singh (1950) and Ahuja (1951), it would be unwise, pending further investigations to deviate from the methods hitherto adopted for the serological identification of *V cholerae* It is reassuring to note in this connexion that in his 1954 article on cholera laboratory diagnosis, Gallut while insisting upon the use of monovalent O sera for tube-agglutination tests considered it legitimate to use for rapid slide tests a serum manufactured by immunization of rabbits with cholera vibrios of either the Ogawa or the Inaba type which had been heated at 100 C for 3 hours.

It has to be noted in connexion with Gallut s work that he evolved a reliable and comparatively simple method of absorbing immune sera for cholera-diagnostic work similar to that used by Venkatraman & Pandit (1938) As stated in his 1949 article, Gallut proceeded as follows

" An agar culture of the vibrios in a Roux bottle is harvested after an incubation at 37° for 18 hours and suspended in 19 ml of normal saline one adds 1 ml of the immune serum and shakes the mixture mechanically for one hour. After the mixture has been kept in the refrigerator for 24 hours, it is centrifuged. One verifies that the serum thus diluted to 1 : 20 is effectively absorbed it should no longer agglutinate the strain used for absorption at a titre of 1 : 100 The agglutination titre for the homologous strain invariably becomes reduced by the absorption, but this reduction takes place at varying proportions according to the antigenic relationships of the strains used The absorbed sera diluted to 1 : 20 if kept in the refrigerator retain a sufficiently high agglutinating titre for several weeks." [Trans.]

A new description of the method of manufacturing type-specific cholera immune sera was given by Burrows & Pollitzer (1958) as follows

" In the preparation of type-specific antiserum by absorption of bivalent or monovalent antiserum with the heterologous serotype H-O antigen, e.g. living or formolized vibrios, may be used. Antiserum may be absorbed undiluted or diluted 1 5 using approximately 1 Roux bottle agar culture of vibrios per ml of undiluted serum or per 5 ml of 1 5 diluted serum. The growth is suspended in the serum by washing it directly off the slant, and is incubated at 37°C for two or three hours, either with constant gentle agitation or with shaking every 15 minutes. The bacteria are removed by centrifugation, the supernatant serum is decanted on to the next agar culture, etc. Usually three such absorptions suffice to exhaust the antiserum of antibody homologous to the absorbing antigen and only rarely are as many as five such absorptions required."

(b) Technique of agglutination tests As has been discussed in Chapter 4 Bandi (1910) recommended the direct use of immune-serum-containing fluid media into which the suspect stools were inoculated for the purposes of cholera diagnosis and similar methods have also been proposed by some other workers, recently for instance by Seneca & Henderson (1949)

However, either these methods have been found unsatisfactory or their usefulness which *a priori* is rather problematic, has not been confirmed through field experiences It therefore remained the generally adopted practice to use the growths of cholera-suspect materials on solid media or subcultures made from such originally inoculated media for the purpose of preparing suspensions for agglutination tests Gardner & Venkatraman (1935) alone recommended the alternative method of obtaining antigens for such tests by growing the suspect organisms for 24 hours in veal broth (pH 8 0) and then killing them by adding, respectively 0.2% of formol and of chloroform It deserves attention however, that in the experience of Burrows et al (1946) formol-saline suspensions of vibrios made from solid media were not inferior in agglutinability to the formolized broth antigen of Gardner & Venkatraman and were preferable in view of the not infrequent sediment formation in the latter type of antigen

As far as the usual method of obtaining material for agglutination tests from solid media is concerned, it is generally agreed that after inoculation the latter ought to be incubated for a period of not less than 18 and not more than 24 hours. To use the growths after a shorter incubation period is not advisable in view of the claim of Friedberger & Luerssen (1905) mentioned in Chapter 4 that then a "pseudo-agglutination" of the organisms under test might prove misleading

The question whether live or killed antigens should be used for the agglutination tests has not been uniformly answered.

The early workers (see for instance Koch, Kirchner & Kolle 1902) invariably used live organisms not only for their orientative tests, made with the aid of hanging-drop preparations but also for tube agglutination. The practice of using live vibrios was also generally recommended by some modern workers—for instance, by Seal (1935) Linton & Seal (1935) and Sugio & Shimomura (1936) and for testing the sera of cholera vaccinated individuals by Brounst & Maroun (1949) and by Gallut & Brounst (1949) — and is of course, universally resorted to for the preliminary identification of cholera suspect colonies with the aid of rapid slide tests.

Gardner & Venkatraman (1935) having to rely for the purposes of their studies solely upon tube agglutination tests, established the important fact that, in contrast to what was the case in the *Salmonella* group formol did not inhibit the O-agglutinability of *V cholerae* and therefore worked with formolized suspensions

In the experience of Burrows et al (1946) it was fully satisfactory to use suspensions of classical cholera vibrios killed by the addition of 0.2% formol for tube-agglutination. However these observers noted that, in the case of the *V El Tor* agglutination tests with live suspensions gave more consistent results than those with formol-killed organisms.

Gallut (1949) working both with live vibrios and with suspensions to which 5 per 1000 of formol had been added, obtained equally good results

with both He stated, however that the formolized suspensions were more practical because they could be kept for one week at least without under going appreciable autolysis. It is noteworthy however that, when dealing again with the technique of tube-agglutination tests in his 1954 instructions for cholera laboratory work, Gallut advocated the use of "preferably not formolized" suspensions. These were apparently also recommended by Ahuja et al. (1950 1951) while Kauffmann (1950) stated that he had resorted to formolized suspensions for his tube-agglutination tests.

As has been discussed before Gardner & Venkatraman were not in favour of using O antigens obtained through boiling of the suspensions for 2 hours for tube agglutination tests, because thus prolonged exposure to heat depressed the O-agglutinability of the vibrios. They added that, as a few experiments had convinced them, exposure of H+O suspensions to 95° 100° C for a few minutes sufficed to remove their H-agglutinability and that formolized broth cultures treated in this manner were "excellent reagents for detecting and measuring O agglutinins" Nevertheless, Gardner & Venkatraman advocated the use of H+O suspensions for cholera-diagnostic work and this procedure has been adopted by all modern workers except Tang, Chu & Wong (1944) who employed *V cholerae* suspensions which had been boiled for two hours for their type identification tests. Still though this technique apparently proved satisfactory for the purposes of Tang and co-workers there can be no doubt that for routine cholera diagnosis H+O suspensions ought to be used in combination with O-agglutinating sera. As has been noted above the latest advice seems to be that live vibrios ought to be employed in preference to formolized suspensions.

The simple technique universally adopted for the performance of rapid slide-agglutination tests consists of placing drops of normal saline and of diluted O-agglutinating serum on slides and distributing material taken with the aid of a loop from cholera suspect colonies first in the saline and then in the immune serum. The appearance of agglutination in the latter which takes place almost immediately should be observed with the aid of a hand lens or preferably under the low power of the microscope A conglomeration of the organisms under test taking place also in the saline control drops which usually indicates a transition of the vibrios into the rough state renders the specimens in question unfit for a rapid presumptive diagnosis.

Since, as has been discussed in Chapter 4 organisms other than vibrios may become paragglutinated by cholera-immune sera, it is indispensable to confirm positive results of slide-agglutination tests through bacterioscopic examinations. The specimens necessary for these can easily be prepared by (*a*) spreading out the drops in which the organisms under test have been distributed, specially the saline drops, in thin layers (*b*) fixing the films thus made, after they have become air-dry by heat and (*c*) staining them with dilute carbol-fuchsin.

It is obvious that bacterioscopic examinations are also essential in cases where slide agglutination tests made with material from macroscopically suspect colonies give a negative result, because otherwise the presence of cholera like vibrios in the stool or water samples examined would be overlooked

Though occasionally lower serum dilutions have been used for rapid slide tests, most modern cholera workers recommend dilutions ranging from 1/50 to 1/100 It is important to note in this connexion that in the experience of Burrows et al (1946)

"Preliminary investigation indicated that the occurrence of low titer agglutinin for the cholera vibrio was common in normal rabbit serums. Titers varied from 1 : 20 to 1 80 in individual serums and pools almost invariably showed a titer of not less than 1 : 50. No normal rabbit serums were found to agglutinate the vibrios to a titer of 1 100 however and this relatively high level was taken as the lower limit of significance."

In view of these observations it would be well to adopt a serum dilution of 1/100 as the standard for rapid slide agglutination tests, even though many workers, including the present writer, obtained fully clear-cut results when using a dilution of 1/50 for a preliminary examination of cholera suspect growths.

The technique of tube agglutination tests may be exemplified by describing the methods adopted for this purpose by the following workers

1 Koch and co-workers (1902) The test sera are diluted with 0.8 % saline (passed two times in succession through hardened paper filters for the purpose of complete clarification) in proportions of 1 50, 1 100, 1 500 1 1000, and 1 : 2000. Loopfuls of the agar cultures to be tested are evenly distributed into 1-ml amounts of these serum dilutions put into agglutination tubes. These are examined after an incubation at 37°C for one hour preferably by holding them in a slanting position and inspecting them with the aid of a weakly magnifying lens from below in the daylight reflected from the ceiling of the room.

Controls must be made with (*a*) the suspect culture and the normal serum of the animal species which served for serum manufacture (*b*) with saline alone and (*c*) with a known cholera culture incubated as long as that under test.

2. As summarized in the 1932 volume of the *Tropical Diseases Bulletin*, Kiribayashi (1931) recommended the following method of tube agglutination, which he claimed to be not only expedient but more sensitive than the ordinarily used procedures

"A series of 10 tubes is set up each containing 2 cc. of 1 per cent. peptone water A dilution of cholera agglutinating serum is made at 1 in 25 with the same 1 per cent. peptone water and 2 cc. of the serum-dilution is added to No 1 of the series of tubes. Successive transferences of 2 cc. of mixtures made in each of the tubes, except the last which remains as a control, give dilutions of cholera serum in peptone water ranging from 1/50 to 1/12,800. A suspicious colony from a plate culture spread with test faeces is suspended in 1 cc. peptone water and one drop added to each of the 10 serial tubes. The tubes are then incubated for 3 hours and readings taken of the resultant agglutination."

3 Gardner & Venkatraman (1935) stated that they had used the agglutination method of Dreyer (1906 1909) as described by Gardner (1931) The tubes were incubated in a water-bath at 51 53°C and readings were taken after 4-5 hours and finally after 18-24 hours. As the two workers added, "the end-point recorded was the last definite trace of agglutination visible with a weak lens by artificial light against a dark background"

4 To ascertain the presence of cholera agglutinins in the population of certain rural areas of India, Read & Pandit (1941) tested the sera, which had been collected in the field and sent to the laboratory in ampoules, in dilutions of 1/25 1/50, and 1/125 against formolized and boiled suspensions of Inaba and Ogawa strains of *V cholerae* Readings were taken after the tubes had been kept for 2 hours in the incubator and for a further 22 hours at room temperature.

5 Tang, Chu & Wong (1944) used for their agglutination tests 24-hour agar cultures of the strains to be examined. After saline suspensions made from these growths had been boiled for 2 hours, they were adjusted in a standard comparator with barium sulfate emulsions to a density of about 2000 millions of vibrios per ml. Dilutions ranging from 1/20 to 1/2560 were made from Inaba and Ogawa monospecific sera, and 0.5 ml of each dilution was mixed in an agglutination tube with an equal amount of the vibrio suspensions. Readings were taken after the tubes had been kept at 56°C for 2 hours and at 37°C for a further 18-24 hours.

6. Referring to the agglutination method they used for the purpose of their investigations, Burrows et al. (1946) stated the following

"The usual method of setting up the agglutination test based on tube-to-tube dilution to give serum dilutions as reciprocals of 2^n is slow and cumbersome and is impractical when large numbers of titrations are carried out. We have, therefore, employed a method which makes possible more rapid and accurate serum dilutions. The tubes are set up in groups of 3 on large boards and saline serum and antigen added in the amounts shown in [the following] table

Serum dilution	*Tube number*	*Saline (cc)*	*Serum (cc)*	*Antigen (cc)*	*Final dilution*
1 : 50	1	0	0.50	0.5	1 : 100
	2	0.25	0.25	0.5	1 : 200
	3	0.40	0.10	0.5	1 500
1 : 500	4	0	0.50	0.5	1 : 1 000
	5	0.25	0.25	0.5	1 2,000
	6	0.40	0.10	0.5	1 5 000
1 : 5,000	7	0	0.50	0.5	1 : 10,000
	8	0.25	0.25	0.5	1 : 20 000
	9	0.40	0.10	0.5	1 : 50 000."

Burrows et al. pointed out that only three pipettes were required for the preparation of these serum dilutions and that dispensing could be facilitated by using burettes.

A further important observation made by these workers was that

"there appeared to be no difference between agglutinations incubated at 37°C and 53°C. The former was preferable since it allowed the routine use of the usual incubator At either temperature agglutination was incomplete in 6 hours but complete at 15-18 hours. The tests were then incubated at 37°C overnight, and were read against a dark background without hand lens"

The technique recommended by Ahuja et al. (1950 1951) for cholera-diagnostic work was as follows

"Agglutination tests are carried out in a waterbath at 52°C with Inaba and Ogawa anti-O agglutinating sera. Young cultures of freshly isolated strains show a tendency to rapid lysis and, if a number of tests are being done at a time, it helps to place each rack in the waterbath as soon as the addition of the culture suspension has been made without waiting for the whole series of additions to be completed. The O agglutination of cholera usually appears very early and a preliminary reading may be taken at the

end of 2 hours, but the final reading is taken next day after the racks have been stood at room temperature overnight. The suspensions should agglutinate at over 50 / 75% of the titre of either Inaba or Ogawa anti-O serum."

Similarly Gallut (1954) prescribed the following agglutination technique

A suspension of an 18-24-hours-old agar culture in 8.5 per 1000 saline and containing about 2000 millions of cholera vibrios per ml is utilized for the tests. The serum dilutions should vary from 1/100 up to the maximum titre of the sera used (Inaba and Ogawa anti-O sera and absorbed monovalent anti-O Inaba and Ogawa sera) Agglutination generally appears quite rapidly so that preliminary readings can be made after the tubes have been kept in the water-bath at 52°C for 2 hours. A second reading is taken after the tubes have been kept at room temperature overnight.

It is certain that tube agglutination tests are altogether indispensable for establishing the diagnosis of cholera in early and sporadic cases. It would also be most desirable to use this elaborate method as a matter of routine during outbreaks. However as exemplified by the experiences of the present writer in the cholera epidemics raging in China during the Second World War which often, with lightning speed involved wide regions or even whole provinces, it may be impossible to make tube agglutination tests with the growths isolated from each patient. In the face of such emergency situations it was often unavoidable to base the final diagnosis of cholera upon the outcome of rapid slide tests combined with the cumulative evidence procured through simple confirmatory tests. Provided that its use was restricted to clinically typical cases, thus simplified procedure appeared to give sufficiently reliable results.

(c) Identification of dissociated cholera vibrios Commenting upon the results obtained in cholera-diagnostic work by various observers in India with O-agglutinating sera (see Gardner & White, 1937) White (1937) stressed that

(a) "Certain variants [of *V cholerae*], selectively produced through the action of cholera phages B to M or combinations of the latter may show a reduced agglutinability with anti-cholera O sera" and

(b) "The rough variant of the cholera vibrio which is selectively produced through the widely distributed A type of choleraphage and which no longer synthesizes the specific polysaccharide responsible for the O-serological reactions of the smooth form, is not agglutinable by O-agglutinating serum raised against the smooth type of *V cholerae*"

White admitted that any doubts which might arise on account of a reduced agglutinability of the cholera vibrios under the action of cholera phages B-M could and should be overcome with the aid of agglutinin absorption tests However in view of the antigenic difference existing between the S and R forms of *V cholerae* it was indispensable for the identification of the latter to resort to special agglutinating sera manufactured with rough cholera vibrios Moreover in view of the instability of the latter in 0 85% normal saline it was necessary for all serological work with rough cholera vibrios to use solutions with a NaCl content of only 0 4% 0 5%.

Ahuja (1951 see also Singh & Ahuja, 1951) also emphasized the necessity of using rough as well as smooth O-agglutinating sera for cholera diagnostic work. Ahuja pointed out in this connexion that the SR variation

"may display different degrees of roughness. Some strains which, according to all accepted criteria, morphological, physical, serological, etc., appear to be smooth cholera vibrios, display the presence in varying degrees of rough element when tested against cholera rough O serum. They even lack the characteristic property that of salt instability in 0 85 per cent sodium chloride, which is considered one of the usual characters of a rough strain. The colonial appearance may be that of a typical smooth vibrio and yet the strain may have varying degrees of rough antigen in its make-up"

As has been described in the fourth chapter Ahuja (1951) and Singh & Ahuja (1951) recommended a test based on the vibriocidal action of guinea-pig serum on rough or partially rough cholera vibrios for the detection of the R state. It has been added that the reliability of this method, which was questioned by Gallut (1953) was reaffirmed by Dudani (1955) Still, there can be no doubt that for the routine purposes of cholera diagnosis agglutination tests with rough O sera are rather more expedient than the quite elaborate procedure of Singh & Ahuja.

Haemolysis tests

The problem of haemolysis and the general principles underlying the carrying out of haemolytic tests, which are indispensable for a differentiation of the classical *V cholerae* from the El Tor vibrios in the strict sense have already received full attention in earlier parts of this book. It seems sufficient, therefore to quote here the methods recommended by Ahuja et al. (1950 1951) for the performance of haemolysis tests with vibrios isolated for the purpose of cholera diagnosis

"The haemolysis test is done by adding 1 ml of 24-hour culture in isotonic Douglas broth to 1 ml of a 5 / suspension of washed sheep or goat erythrocytes, incubating the mixture at 37°C for 2 hours followed by overnight storage in the cold room. It is essential to make sure that the erythrocytes used in the test are not fragile in 0 65 / saline. Alternatively a saline suspension of a 24-hour growth on nutrient agar standardized to contain 8,000 million organisms per ml, may be used. To 1 ml of this vibrio suspension is added 1 ml of a 3 / suspension of washed erythrocytes and the mixture [is] treated as above. Krishnan, who recently made a detailed study of the various factors involved in the tests, has shown that more consistent results may be obtained by using broth cultures in preference to saline suspensions of agar cultures and that a 24-hour growth in broth is *preferable to a 48- or 72-hour growth.*"

It would be most desirable if the method ascribed in this statement to Krishnan alone but actually recommended by Krishnan & Gupta (1949) (see Chapter 3 page 148) were to be adopted as standard It is important to note that in the experience of these two workers the use of the more easily available sheep erythrocytes was preferable to that of goat erythrocytes

Bacteriophage tests

In the course of a lecture on the problems of cholera bacteriology Finkelstein (1931) reported that according to unpublished observations by Clark cholera and cholera like vibrios did not behave uniformly when subjected to the action of a choleraphage which had been supplied by Morison Though 30 *V cholerae* strains were lysed by this phage, 8 were not, whereas out of 23 cholera like strains 11 were lyso-sensitive and 12 resistant.

Combiesco-Popesco & Wisner (1933) on the other hand, found all the 15 cholera like strains they were able to examine resistant to the action of a choleraphage received from Egypt, while not less than 94% of their 67 cholera strains were lysed by this.

Commenting upon these observations, which had been made in his laboratory Cantacuzène (1933) pointed out that

"Lysosensitivity by phage appears to be a phenomenon most often, *but not invariably* linked with the authenticity of the cholera strains. One ought to consider it as a phenomenon of great probability but not of certitude" [Trans.]

The limited diagnostic value of bacteriophage tests was also admitted by Seal (1935) who made in this connexion the following statement

"Study regarding the bacteriophage is yet far from complete. New phages are still being discovered and added to an already long list Different vibrios have been found to behave differently with these phages. They may be lysable by one or more of these phages or may be completely resistant to all. A -phage lysability has been held as a criterion for complete smoothness, but smooth vibrios have later on been found by Pasricha and his collaborators [1932b], which are not A-phage lysable The difficulty specially arises with those vibrios which are phage resistant. Non-agglutinable vibrios obtained from cholera cases, convalescents, carriers, water etc., have often been found phage resistant or lysable only by few or mixed phages. On the whole, phage lysability may be utilized as an additional confirmatory test and this is always done nowadays in all experimental work."

A consideration of these statements and also of the great difficulty of maintaining suitable phage strains in readiness makes it clear that tests with choleraphages are of no practical value in cholera laboratory work. It is not surprising, therefore that no reference to their use has been made by any modern authority

Confirmatory Tests

Tests for roughness

Before dealing with the tests customarily used for confirming the laboratory diagnosis of cholera, it is indicated to refer to two simple methods which have been recommended for the detection of the rough state of microbial organisms including the *V cholerae*—namely tests with Millon s reagent and with trypaflavine

Millon's reaction According to the standard works on organic chemistry, the reagent of Millon (1849) which is widely used in chemical work for the detection of various substances containing the hydroxy phenyl group, including tyrosine, phenol and thymol, is prepared by (*a*) dissolving 1 part of mercury in 2 parts of strong nitric acid, (*b*) adding 2 volumes of water, and (*c*) decanting the clear fluid after standing. The reagent seems to have been used first for bacteriological work by White (1929) in the course of a study on the smooth and rough races of intestinal bacteria. White stated in this connexion that

"When a large loopful of rough growth is emulsified in approximately 3 c.c. of water and about 1 c.c. of Millon's solution is added thereto, the bacilli are at once clumped and, on boiling the mixture, collected in dense masses and quickly assume the deep red-pink coloration of the positive test. Under the same conditions typically smooth bacilli of the *Salmonella*, colon and dysentery groups are relatively little affected the bacilli remain dispersed and the colour of their suspension does not deepen during a few minutes of boiling beyond a yellowish or ochre tint. The deposit which settles on standing commonly varies in colour from yellow to ochre occasionally it is pale pink—a variation probably related to the degree of smoothness— in all cases it is fine and readily dispersible.

"Smooth bacilli from which the soluble specific non-protein factor has been extracted (*e.g.* by prolonged boiling with dilute acid) or the proteins thrown down by acid from a solution of smooth bacilli in NaOH solution react precisely like fresh rough bacilli and it would seem to be an inevitable conclusion that in the smooth organism the soluble specific carbohydrate intervenes—probably mechanically—between the tyrosin-containing complex and the reagent."

Taking advantage of tests with Millon's reagent in the course of their work on cholera bacteriophage Asheshov et al. (1933b) found that these compared favourably in reliability as well as in ease of application with other procedures used for detecting the roughness of *V cholerae* cultures, including observation of the appearance of the colonies, the character of growth in fluid media, the influence of NaCl concentration, and the type of agglutination. Asheshov et al used for the purposes of their bacteriophage work the following modified method of preparing Millon's reagent

Solution of 1 part of metallic mercury in 2 parts by weight of nitric acid of 36° according to Baumé's scale was done in a fume chamber or in the open air because during this process dense fumes of nitrous oxide are given off After dissolution had become complete, the green liquid obtained was diluted with 2 parts of water and then poured into a large photographic dish, where it was left to aerate for about 24 hours.

To make tests with this reagent, Asheshov et al proceeded as follows

"A loopful of 24 hours growth of microorganisms on agar is emulsified in 2 c.c. of tap-water 0.2 c.c. of Millon's reagent is added, the tube is left for about one minute, and then heated nearly to boiling point. The heat is maintained for about one minute more, but without boiling."

Commenting upon their method, Asheshov et al stressed that their well-aerated reagent did not produce a red coloration when acting upon rough vibrios. This was desirable because in their experience the addition of non aerated Millon's reagent even to smooth cholera cultures led to the

appearance of a red colour owing to the presence of extraneous organic matters as well as of a few rough elements When the aerated reagent was used, reliance had to be placed upon the presence or absence of two other phenomena thus described by Ashesho v and his colleagues

"1 *Flocculation.* With pure rough culture this appears even without heating, the flocculi slowly falling to the bottom On heating, the flocculi coagulate partly floating on the froth, partly falling to the bottom, clearing the liquid more or less completely The more complete the coagulation and the clearer the liquid the more rough is the culture.

"2 *Creeping* After heating, it will be observed that a film, more or less granular is creeping up along the wall of the test tube above the surface of the liquid. It consists of coagulated vibrios which float on the surface of a thin layer of the liquid adhering to the wall. The more marked the phenomenon the more rough is the culture."

Trypaflavine reaction Trypaflavine first considered by Alessandrini & Sabatucci (1931) as a means of distinguishing between supposedly different *Brucella* species, was recognized by Pampana (1931 1933) as a reagent suitable for a differentiation between the smooth and rough forms of one and the same bacterial species.

Describing his technique Pampana (1933) stated

"The reagent consists of a 1:500 solution of *trypaflavine* in normal saline. A drop of the solution is put on a slide. Close to the drop but not in the drop we depose a minute fraction of a loopful of the bacterial colony to be examined. We then flame the loop, and, when it is cool again, we moisten it gently with the trypaflavine and gradually emulsify the material on the slide. Finally we mix it with the whole droplet of trypaflavine solution. If the colony contained the "*R*" variant, agglutination takes place immediately or within a few seconds. The reaction is very easily read, the more so if the surface of the slide is illuminated by oblique light against a dark background."

Pampana added that his test could also be performed by mixing equal amounts of 1/500 trypaflavine solution and of the bacterial suspension to be examined in a test tube, but found this procedure less sensitive than the above-described " drop-agglutination " method.

Working with 100 vibrio cultures, including El Tor and cholera like vibrios besides classical *V cholerae* strains Popesco-Combiesco & Soru (1934) confirmed the value of the latter test, with the aid of which they were able to distinguish between smooth, SR, and rough organisms. The former two types produced a uniformly turbid growth in broth the latter a granular growth. Results obtained with the acid agglutination method of Dambo-viceanu (1933) (see Chapter 4 page 280) paralleled those of Pampana s test, also permitting a classification of the vibrios into three groups However in contrast to the trypaflavine reaction, Damboviceanu s method was too elaborate to be of practical value

Bhaskaran (1953) who had the opportunity of examining variant strains of *V cholerae* obtained through cultivation on Aronson-type media (see page 559 above) with rough O-agglutinating sera as well as with Millon s and Pampana s reagents, commented thus on the results of these combined tests

" From all the strains of *V cholerae* rough variants were obtained during cultivation in B C. agar [i.e., basic fuchsin/sodium-carbonate agar] which gave positive results with

Millon s and trypaflavin tests and agglutinated wholeheartedly with rough O serum. Further every grade of intermediate condition between smoothness and roughness was seen to exist. At one end of the scale were the typically rough strains which reacted only with rough O sera, while not being clumped by smooth O sera at as low a dilution as 50 The majority of the variants, however represented intermediate stages between smoothness and roughness. These variants, while satisfying Millon s and trypaflavin tests, agglutinated with rough as well as the homologous O sera. At the other end of the scale were a few smooth strains which were recovered unaffected after cultivation in B.C. agar "

As far as one may judge from these findings made with artificially dissociated variants of *V cholerae* tests with Millon s and Pampana s reagents were as reliable for detecting the presence of rough elements in these growths as those with rough O-agglutinating sera. However attention has been drawn above (page 588) to the observations of Ahuja (1951), who found, among the cholera strains at his disposal, some reacting with rough O serum even though they lacked all other characteristics indicating roughness. In view of this and more still because, as confirmed by Bhaskaran, cholera strains do occur which react solely with rough O sera, there can be no doubt that tests with these have to be resorted to in cholera diagnostic work regardless of whether tests with Millon s and Pampana s reagents are utilized as well

Cholera-red reaction

It is of historical interest to note that the technique now generally adopted of performing the cholera red reactions with organisms cultivated in peptone water was not used by the pioneer workers Poehl (1886) who first recorded that addition of hydrochloric acid to *V cholerae* growths led to the appear ance of a red " pigment " worked with Koch s nutrient gelatin. This was also used by Brieger (1887) while Bujwid (1887) who independently described the reaction, resorted to cultivation in broth. However Dunham (1887) systematically searching for an optimal technique to produce the cholera red reaction recommended the use of 1% peptone water with which he said he obtained positive results after cultivation for four hours or once even after an incubation of only three hours. Dunham noted, on the other hand, that gelatin stab cultures of *V cholerae* gave a cholera red reaction only after they had become entirely liquefied, whereas the presence of even inconsiderable remnants of undissolved gelatin led to the appear ance of a brown coloration.

Another important proposal made by Dunham, which has been adopted by modern observers (see for instance Ahuja et al. 1950 1951 and Gallut, 1954) was to use concentrated sulfuric acid in place of hydrochloric or other acids for the performance of the cholera red test.

As already alluded to above (see page 576) the early observers inclined to the belief that a typical cholera red reaction was given solely by *V cholerae* and that consequently a positive outcome of such tests sufficed for the identi

fication of this organism This view was expressed in particular by Bujwid (1888) who reached the now quite amazing conclusion that by (a) cultivating material from cholera suspect stools in 10 ml of 2% peptone water, (b) taking after 24 hours a loop from the surface of the culture for subcultivation in the same medium (c) successively making in this manner two further peptone water subcultures, and (d) using the third subculture for the cholera red test one was able to establish the laboratory diagnosis of cholera without the aid of a microscope

However the belief in the specificity of the cholera red reaction was soon discredited by observations showing that it was also given by cholera like vibrios, such as the *V metchnikovi*, isolated by Gamaleia (1888) from fowls and water vibrios such as the *V berolinensis* (Neisser 1893), the *V danubicus* (Heider 1893), and, according to Prausnitz (1903), the majority of the strains isolated from water samples in Hamburg. Hence as the last mentioned observer maintained with much reason the cholera red test was mainly valuable as an easy means of ruling out the cholera nature of vibrio strains which reacted negatively whereas a positive result obtained in the case of strains morphologically and culturally identical with *V cholerae* was not conclusive

It was sometimes claimed that the nitroso-indole reaction if done with one and the same strain upon successive occasions was apt to give variable results (see for instance, Pottevin 1913 and summary by Pollitzer, 1934) but, as far as the present writer can judge such differences were of a quantitative rather than a qualitative nature It is however of the utmost importance to realize that for various extrinsic reasons cholera red tests done with authentic strains of *V cholerae* may give falsely negative results

In the first place as was recognized by Bujwid (1887) it is necessary to use pure cultures of the organisms under examination for the performance of the tests, because the presence of extraneous organisms is apt to interfere with the reaction Tobey (1908) and Logie (1913) showed that in particular the presence of nitrite-destroying organisms like *E coli* exerted such an untoward influence The direct use of cholera stools for cholera red tests, even though it may lead to positive results is therefore inadvisable

Another indispensable prerequisite for the proper performance of cholera red tests is the use of suitable media Experience has shown that not all brands of peptone are adequate in this respect, because they may be lacking in tryptophane which is indispensable for the formation of indole (Mackie 1929c) A proper content of the media in nitrates is also of crucial importance Bleisch (1893) who made a profound study of this question maintained that besides avoiding too low a nitrate content it was also essential to guard against an excess of nitrates or preformed nitrites He considered broth media apt to show an inconstant composition to be altogether unsuitable substrates for cholera red tests and insisted upon the use of specially prepared 2% peptone water media to which exactly deter

mined amounts of diluted potassium nitrate solution had been added to bring the nitrate content to a proper level. Ample practical experiences have shown, however that—provided that they had been manufactured with suitable brands of peptone—the peptone water media ordinarily used for cholera laboratory work prove reliable for the performance of cholera red tests as well Their suitability may be simply ascertained by growing a known cholera red positive strain of *V cholerae* in them for 24 hours and then adding one drop of strong sulfuric acid per ml of the medium (see for instance Taylor Pandit & Read, 1937) However even if the lot of peptone used has been found suitable through such preliminary tests, it is advisable always to inoculate a control tube with a known nitroso-indole positive culture of *V cholerae* when making cholera red tests with unknown vibrio strains

Tests for indole

As has been stated in the third chapter tests for indole are of no differential diagnostic value in cholera laboratory work because (*a*) in addition to intestinal bacteria belonging to other genera, many cholera like strains as well as *V cholerae* show evidence of indole production and (*b*) naturally all cholera red-positive vibrio strains are indole producers. However as is to be expected as well, cholera like strains which prove negative in nitroso-indole tests do not react uniformly when being examined for indole formation, yielding either negative or positive results (Taylor Pandit & Read, 1937)

As far as could be ascertained, former cholera workers followed the method introduced by Böhme (1901) to demonstrate the presence of indole in their cultures (see for instance Mackie 1929c)

This method consisted of the use of two reagents composed as follows

(1)	*p*-dimethylamidobenzaldehyde	4 parts
	96 / ethanol	380 parts
	Concentrated hydrochloric acid	80 parts
(2)	Potassium persulfate in saturated aqueous solution (used as oxidant)	

Böhme s procedure was to add to about 10 ml of a broth culture of the organisms to be tested, first, 5 ml of solution 1 then the same amount of reagent 2, and to shake well. The presence of indole was indicated by the appearance of an intense red colour becoming visible at once or within a few minutes.

Taylor Pandit & Read resorted to a modified procedure (*a*) using 24-hours-old peptone water cultures of their vibrio strains instead of broth cultures (*b*) superimposing Böhme s reagent 1 on the culture fluids instead of admixing it and (*c*) omitting the addition of potassium persulfate solution. In their experience slight heating was useful to hasten the appearance of a positive reaction.

Animal experiments

The value of animal experiments for the differentiation of cholera from cholera like vibrios was emphasized by Koch (1893) He insisted in this connexion that, in order to obtain reliable results, it was necessary to work with material from agar cultures and not from fluid cultures. A dose of one loop (about 1.5 mg) of the former growths, suspended in 1 ml of broth, was adequate for the intraperitoneal infection of guinea pigs weighing 300-350 g, but increased doses had to be used for heavier animals. Maintaining that this mode of experimentation invariably led to the appearance of a typical collapse and eventually to the death of the animals Koch stated that

"Since one or a few well-developed [cholera] colonies are able to furnish sufficient material for an animal experiment the great value of making agar cultures early becomes evident. One must rate the method of animal experimentation highly because in analogy with the cholera-red test, it makes manifest a property possessed exclusively by the cholera bacteria. Among all curved i.e. spirilla-like, bacteria which come into question in cholera laboratory work, so far none has been found which produces in the above mentioned dose symptoms even remotely similar to those of the cholera bacteria." [Trans.]

A determined stand against these postulations of Koch was taken by Gruber (1894) who denied the specificity of the method of animal experimentation devised by Koch and moreover stressed that the virulence of cholera cultures as well as the susceptibility of the test animals was variable

Prausnitz (1903) besides suggesting that under certain circumstances, e g., through immersion in water for some time, the cholera vibrios might lose their virulence, laid greater stress upon the fact that many of the cholera like strains isolated from water samples at Hamburg had shown a high pathogenicity for guinea pigs

Subsequent observations have supported the views of Gruber and Prausnitz rather than those of Koch It is true that there is no great likelihood of meeting avirulent strains among the cholera vibrios isolated from the faeces of patients in the acute stage of the disease and that consequently negative results of animal experiments obtained under these conditions speak strongly against the presence of *V cholerae* However a positive result obtained through inoculation of test animals with vibrios isolated from the patients stools cannot be considered conclusive It is obvious, moreover that it would be altogether impossible to make routine use of animal experimentation during a major outbreak of cholera To resort to this method when dealing with early or sporadic cases would be within the realm of practical policy but, as far as the present writer can judge it would be better then to use the available guinea pigs for Pfeiffer s test rather than for simple intraperitoneal inoculation.

mined amounts of diluted potassium nitrate solution had been added to bring the nitrate content to a proper level. Ample practical experiences have shown however that—provided that they had been manufactured with suitable brands of peptone—the peptone water media ordinarily used for cholera laboratory work prove reliable for the performance of cholera red tests as well. Their suitability may be simply ascertained by growing a known cholera red positive strain of *V cholerae* in them for 24 hours and then adding one drop of strong sulfuric acid per ml of the medium (see for instance, Taylor Pandit & Read 1937) However even if the lot of peptone used has been found suitable through such preliminary tests, it is advisable always to inoculate a control tube with a known nitroso-indole positive culture of *V cholerae* when making cholera red tests with unknown vibrio strains

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Carbohydrate tests

From the exhaustive description of the saccharolytic effects produced by cholera and cholera like vibrios in Chapter 3 it will be gathered that for the practical purposes of cholera diagnosis it suffices to make tests with only three sugars, namely saccharose, mannose, and arabinose. It has been shown that with very few exceptions strains which failed to acidify the former two substances or acidified arabinose were not cholera vibrios. At the same time however it has to be stressed that these charac teristic reactions were given not only by *V cholerae* but also by a consider able number of cholera like strains

As has been suggested in the third chapter it would be well to adopt the method of Heiberg (1934) as standard when making tests with these three sugars The procedure of this worker was (*a*) to grow the strains to be tested in peptone water into which the various carbohydrates had been incorporated at a concentration of 0 5% (equivalent—according to Gallut, 1954—to 3 drops of a 30% solution of the sugars per 10 ml of the medium) (*b*) to add a few drops of a 0.2 per 1000 solution of bromothymol blue in ethanol as indicator and (*c*) to take initial readings after an incubation of not more than 20 hours at 37° C so as to be able to distinguish between rapid and late acidifications—an essential distinction for diagnosis. As has been noted, Heiberg worked with peptone water media of comparatively low alkalinity (pH 8 0-8 4) It would be preferable for the sake of uniformity and expediency to use peptone water of a pH of 9.2 for these tests as well as for cholera laboratory work in general (see page 536 above)

Voges Proskauer reaction

The important results which can be obtained through an examination of classical cholera vibrios, El Tor vibrios, and cholera like vibrios with the Voges Proskauer reaction (originally described in 1898) as well as the modern technique preferable for carrying out this method have been fully dealt with in Chapter 3 While it had to be admitted that it was impossible to distinguish with the Voges-Proskauer reaction alone between cholera and cholera like vibrios attention could be drawn to the cardinally important conclusions reached by Taylor Pandit & Read (1937) when considering the results of such tests in combination with those elicited with the aid of the cholera red reaction and fermentation tests with saccharose mannose and arabinose As these workers found, it was possible

" on biochemical evidence alone, to obtain presumptive diagnosis of the serology of the typical *V cholerae* if it gives fermentation reactions of Heiberg type I [i.e., acidification of saccharose and mannose but not of arabinose], is cholera red positive and negative to the modified V P [Voges-Proskauer] test, it is very probably an agglutinable vibrio "

From the foregoing, it transpires that a typical result of such combined tests goes a long way to support a presumptive diagnosis of cholera arrived at through rapid slide agglutination

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Chapter 8

CLINICAL PATHOLOGY

Loss of Fluids and Salts in the Evacuations

In view of the fact that evacuations from the gastro-intestinal tract brought about by a local action of the endotoxin of *V cholerae* form as a rule the earliest and one of the most spectacular signs of clinically manifest cholera infection it is not surprising that investigations on the character of the stools and vomits voided in this disease were commenced as soon as the appearance of outbreaks in Europe offered adequate opportunities for study The often truly amazing copiousness of these evacuations, already commented upon by observers in the East (see page 101 of the summary by Rogers, 1921) was confirmed by for instance, Loder (1831) and Marcus (1832) who witnessed the 1830 epidemic in Moscow Moreover the physical and chemical properties of the cholera stools and vomits were investigated by several of the early workers, such as Hermann (1832) during the epidemic just mentioned and Wittstock (1831) in Berlin Both noted an invariably alkaline reaction of the characteristic cholera stools. The specific gravity of these varied according to the latter observer from 1 0073 to 1 0082 and their salt content appeared to be considerable one specimen of 2000 grains yielding 26 grains of salt from a total of 44 grains of solids

The cholera vomits examined by Hermann and Wittstock varied somewhat in character Those voided at the onset of the disease showed a somewhat higher specific gravity (up to 1 014) and an acid reaction while those obtained later and resembling in aspect the rice watery stools had a specific gravity of only 1 003-1 006 and were alkaline The sodium chloride content of the vomits was found to be markedly below that of the faeces

Results of further examinations of cholera stools were recorded towards the end of the second cholera pandemic by Parkes (1847) Becquerel (1849) and by Schmidt (1850) As quoted by Rogers (1921) the first mentioned worker found that the rice watery stools of cholera patients contained 0 5% 1% of salts but only very little protein—an observation also made by Becquerel and by Schmidt. Becquerel confirmed the observation of Wittstock that the cholera stools contained only a small proportion of solids

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(1% 2%) the NaCl content of which varied from 3 to 7 per 1000 The presence could be demonstrated, not only of sodium chloride but also of ammonium carbonate and sodium phosphate as well as of small amounts of potassium chloride and of traces of creatine and uric acid Like Schmidt he was inclined to consider the rice watery stools of cholera patients as a true transudate analogous in character to the fluids found in instances of hydrocephalus or hydrocele

Examining cholera vomits Schmidt noted a lesser NaCl content in these than in the stools but found them to contain somewhat larger amounts of ammonium carbonate and urea.

Turning attention to modern observations, reference has to be made first to those of Rogers (1909a 1911 1921) who stated that in his experience the average chloride content in rice watery cholera stools was 0 53% while there was only a very small amount of chlorides in the vomits of the patients

A careful study of the chemical constituents of the stools of 31 cholera patients with the aid of modern methods was made by Ghosh & Chakraborty (1940) Summarizing the results which they had recorded in tabular form the two workers stated that

"The stools in all cases were highly alkaline The average alkalinity has been found to be nearly equivalent to 50.7 c.c. of N/10 HCl per 100 c.c. of stool. The average percentage of sodium chloride was found to be 443.2 mg. Protein, non-protein nitrogen, ammonia, phosphorus, sulphur and carbonate contents per 100 c.c. of stool were found to be 0.95 g., 36.9 mg., 9 1 mg. 57.5 mg. 14 1 mg. and 0 081 g., respectively The percentage of total solid material has been found to be 2.05 g. The tests for occult blood were positive in all the samples of stools examined."

Commenting upon these findings, Ghosh & Chakraborty (*a*) stressed the alkaline reaction of the stools which in their opinion favoured the formation of cholera toxin, and (*b*) drew attention to the elimination of considerable amounts of alkaline bases as well as of chlorides in the dejecta. These losses were bound to disturb the osmotic balance and to lead to acidosis.

In an interesting article on hypochloraemia in cholera, Banerjee (1941a) made the following statements regarding the problems at present under review

(1) The total volume of stools passed during successive 24-hour periods by 20 cholera patients varied from a minimum of 500 ml to a maximum of 5000 ml per day while the sodium chloride content varied from a minimum of 110 mg per 100 ml to a maximal figure of 865 mg or 0 865 %. Since the quantity of the stool sample with this maximal saline content was 4000 ml, the loss in chlorides during 24 hours amounted in this instance to 34 6 g.

(2) The amount of vomitus, determined in the case of four patients only averaged 3325 ml per 24 hours, while the sodium chloride content per 100 ml was at an average 293 mg. Hence "the total excretion of NaCl through vomiting during 24 hours had been more than 9 7 grammes in an average case of cholera "

As stated in the 1941 report of the Indian Research Fund Association investigations by Panja had shown that the sodium chloride content of cholera vomits varied from 66 mg to 821 mg per 100 ml while their pH though varying from 2 4 to 8 4 was usually between 6 0 and 7 0 According to a statement in the 1946 report of the Indian Research Fund Association the chloride content of 100 typical cholera stools (average pH 7 8) was at an average 0 431 g.

In the course of a study on the fluid balance in cholera made during the 1947 Egyptian outbreak Safwat & Adham (1948a) found that

"There was a tremendous water loss in the vomit and stools. Figures showed that as much as 10 litres were lost by many cases in the course of 24 hours, while some figures indicated that as much as 15 litres were lost by other cases. Though the loss in the vomit was great, yet in the majority of cases the loss in the stools was much greater"

The two workers apparently did not determine the salt content of the evacuations but they came to the general conclusion that cholera presented a combination of water and salt depletion

El-Ramli (1948) stated in a valuable clinical study on 689 cholera patients isolated in a fever hospital that the amounts of fluid lost by the sufferers in their stools and vomits varied considerably but that instances were met with where they lost one litre of fluid in a single faecal discharge and 4 litres or more in 24 hours The amount of vomit could reach 800 ml upon a single occasion and it was noted that one patient, who had been given 10 litres of fluid by mouth vomited as much as 7 litres in one day The degree of dehydration ultimately developing was not invariably proportional to the fluid loss some sufferers showed marked signs of dehydration even though they had lost comparatively little fluid while others, who had voided much larger amounts of fluid, became but mildly dehydrated The chloride content of the stools was found to vary from 221 mg to 750 mg per 100 ml that of the vomits from 48 mg to 351 mg.

As summarized in the *Tropical Diseases Bulletin*, Saha & Das (1952) arrived through an examination of a series of cholera stools at the following average figures

"Specific gravity—1,010 sediment—9.8 per cent. reaction—pH 7.86 total protein—0.26 gm. per cent. sodium—286.2 mgm per 100 ml. potassium—74 6 mgm. per 100 ml. chlorine ion—268 mgm. per cent. inorganic phosphate as PO_4—3 6 mgm. per cent bicarbonate—68.2 ml. of CO_2 per cent."

While unable to detect Evans blue in the stools examined 1 2 hours after intravenous injection, Saha & Das found thiocyanate in every specimen tested in this respect, though in a somewhat lesser concentration than corresponded to the amount injected into the blood stream The conclusion they reached was that the fluid part of cholera stools resembled physically and chemically the plasma minus its protein and was of the nature of a transudate the intestinal mucosa acting as a semipermeable membrane

The two workers thus supported the postulations made more than a century before by Becquerel (1849) and Schmidt (1850)

In order to arrive at an evaluation of the findings recorded above it is necessary to pay attention to the observations made within recent years on water and salt depletion in general Experimental findings by Kerpel Fronius (1935) and tests on normal men by Nadal and co-workers (1941) as well as recent clinical observations have clearly shown that, besides a mixed type of depletion, either pure water or pure salt depletion may occur under varying conditions Marriott (1947) exhaustively dealing with this and related problems stated that

(*a*) *Pure water depletion* occurs when the intake of water is stopped or greatly diminished without a significant loss of salt. This condition may develop in shipwrecked individuals in instances of dysphagia, or in coma.

Lack or deficiency of water intake without a corresponding loss of salt renders the extracellular fluid hypertonic As a result water is sucked from the cells so that dehydration takes place mainly at the expense of the cellular fluid.

(*b*) *Pure salt depletion* due to abnormal losses of sodium and chlorine in the presence of an adequate water intake may develop in various pathological conditions, such as severe vomiting or diarrhoea, presence of a biliary or intestinal fistula, in Addison s disease and in heat exhaustion due to severe sweating combined with an abundant intake of salt free fluids

As a result of salt depletion the extracellular fluid becomes hypotonic and greatly diminished in amount because the kidneys excrete water in an attempt to maintain isotonicity There is also a relative shift of fluid from the tissues into the plasma. However a condition of oligaemic circular failure or shock is apt to follow and the decreased blood volume combined with an increased viscosity of the blood leads to death

As a secondary effect disturbances of the acid base balance due to an asymmetrical loss of sodium or of chlorine may develop Thus alkalosis may result in instances of severe vomiting, acidosis in the case of violent diarrhoea.

(*c*) *In mixed water and salt depletion* the patients show the circulatory and other features due to salt depletion, but they are also thirsty and show early oliguria, as is characteristic of water depletion.

While there can be no doubt that cholera falls into the category of diseases with a mixed water and salt depletion it is equally true that, as aptly summarized by Shattuck (1951)

" In cholera loss of water and of salts occurs in amounts which vary from time to time in the individual case and which differ in degree in relation to the severity of the symptoms "

One must admit however that the evidence regarding a manifest influence exerted by a differently proportioned loss of water and electrolytes

respectively on the clinical picture of cholera is contradictory as well as scanty

It has to be noted in this connexion that Ghanem & Mikhail (1949) studying the clinical pathology of cholera came to the conclusion that in this disease a loss of water as well as of salts was responsible for the signs of dehydration, but that the former was more important, since no special clinical manifestations could be ascribed to salt depletion alone.

Massias (1938) on the contrary asserted that chloropenia played a more important role in cholera than water depletion, many of the grave features of the disease being due to hypochloraemia Similarly Rogers (1952) referred to particularly severe cholera cases in which a loss of salts from the blood preponderated over that of fluid and in which, therefore the chloride content of the blood could fall below 0 85%, haemolysis taking place in the circulation in extreme cases

More significant perhaps than these statements are observations recorded by Banerjee (1939a) who maintained that in cholera two special types could be distinguished from the usual "straightforward" form—namely (*a*) a "renal failure type" in the causation of which hypochloraemia presumably played a more important role than simple dehydration and (*b*) a "vasomotor failure type" in which one might venture to suggest, water loss was of preponderant importance

It would certainly be most desirable to obtain further evidence regarding the occurrence of these special types of cholera and their clinical pathology

Blood Changes

Introductory remarks

In explanation of the fact that investigations on the blood changes observable in cholera attracted early attention, Liebermeister (1896) aptly stated that

> "The first European cholera epidemics occurred at the time of the predominance of haemopathology when it was believed that a chemical investigation of the blood would furnish particularly important clues for the nature of the diseases. Moreover at that time still, most abundant use was made of blood-letting and since this method was also often applied in cholera, sufficient material was available for exact investigations. In fact one found in the blood of cholera sufferers at the acme of the attack and in the asphyctic stage alterations surpassing in importance those met with in any other acute disease. While it was not possible to obtain the hoped-for insight into the nature of the disease, these older investigations remain valuable, because they furnish important clues for an appreciation of the morbid symptoms." [Trans.]

That the early studies by workers like Hermann (1832) in Moscow O Shaughnessy (1831 32a, 1831 32b 1832) and others in Great Britain, Le Canu (1832) in France, Wittstock (1832) and Rose (1833) in Berlin were valuable indeed is well illustrated by a preliminary communication

The two workers thus supported the postulations made more than a century before by Becquerel (1849) and Schmidt (1850)

In order to arrive at an evaluation of the findings recorded above, it is necessary to pay attention to the observations made within recent years on water and salt depletion in general Experimental findings by Kerpel Fronius (1935) and tests on normal men by Nadal and co-workers (1941) as well as recent clinical observations have clearly shown that, besides a mixed type of depletion either pure water or pure salt depletion may occur under varying conditions. Marriott (1947) exhaustively dealing with this and related problems stated that

(*a*) *Pure water depletion* occurs when the intake of water is stopped or greatly diminished without a significant loss of salt. This condition may develop in shipwrecked individuals, in instances of dysphagia or in coma.

Lack or deficiency of water intake without a corresponding loss of salt renders the extracellular fluid hypertonic As a result water is sucked from the cells so that dehydration takes place mainly at the expense of the cellular fluid.

(*b*) *Pure salt depletion* due to abnormal losses of sodium and chlorine in the presence of an adequate water intake, may develop in various pathological conditions, such as severe vomiting or diarrhoea, presence of a biliary or intestinal fistula, in Addison s disease and in heat exhaustion due to severe sweating combined with an abundant intake of salt free fluids.

As a result of salt depletion the extracellular fluid becomes hypotonic and greatly diminished in amount because the kidneys excrete water in an attempt to maintain isotonicity There is also a relative shift of fluid from the tissues into the plasma. However a condition of oligaemic circular failure or shock is apt to follow and the decreased blood volume combined with an increased viscosity of the blood leads to death.

As a secondary effect disturbances of the acid base balance due to an asymmetrical loss of sodium or of chlorine may develop Thus alkalosis may result in instances of severe vomiting, acidosis in the case of violent diarrhoea.

(*c*) *In mixed water and salt depletion* the patients show the circulatory and other features due to salt depletion, but they are also thirsty and show early oliguria, as is characteristic of water depletion

While there can be no doubt that cholera falls into the category of diseases with a mixed water and salt depletion it is equally true that, as aptly summarized by Shattuck (1951)

"In cholera loss of water and of salts occurs in amounts which vary from time to time in the individual case and which differ in degree in relation to the severity of the symptoms"

One must admit, however that the evidence regarding a manifest influence exerted by a differently proportioned loss of water and electrolytes

respectively to 78 43—73 11—74 93—76 07 / Thomson noted in his cases a still more marked decrease of the water content whereas he determined that of normal blood at 78.39 /, he found in two cholera patients 66.121 and 67 94 / respectively " [Trans]

Further observations on the specific gravity of the blood and plasma

As Rogers summarized in 1921 he found that

" In the acute stages of the disease [cholera] the specific gravity [of the blood] nearly always varies between 1060 and 1068 rarely reaching as high as 1072 in natives of India, in whom the normal figure in health is about 1054 I have seen it as high as 1076 but only in extremely severe cases. The most common point is about 1063 to 1065 which means a loss of about half the fluid from the blood and is nearly always accompanied by general symptoms indicating the necessity for transfusion."

Observations made by subsequent observers may be tabulated as follows

Author	*Findings*
Loh & Tai (1936)	Found, measuring the specific gravity according to the method recommended by Rogers (1911 1921) in 13 cholera patients before saline treatment had been given, values ranging from 1.056 to 1 069 (average 1.064) After saline infusion the specific gravity of the blood became lower than normal (average 1 053) In the opinion of Loh & Tai this drop was partly due to the replacement of the body fluids of high specific gravity by saline of lower specific gravity The blood specific gravity of 16 patients examined 3 or more days after onset of the disease varied from 1 046 to 1 068, but was 11 times 1 056 or less.
Banerjee (1938)	Stated that his numerous cholera patients who required saline infusions had blood specific gravities varying from 1 063 to 1 070.
Ghosh & Chakraborty (1940)	The blood specific gravity of 31 cholera patients examined at the time of their admission was found to range from 1.062 to 1 066.
Safwat & Adham (1948a)	Determined the specific gravity of the blood in 11 cholera patients by the copper sulfate method (Phillips et al., 1943) The values ascertained before treatment ranged from 1 068 to 1 075 as against normal standards of 1 052—1 055 in Egyptians and 1 056—1 058 in Europeans
El Ramli (1948)	Found, also using the copper sulfate method blood specific gravities in cholera patients ranging from 1 050 to 1.078 It is noteworthy that a normal specific gravity of the blood was found in 2.2 / of 124 patients with severe dehydration, 2.2 / of 83 patients with moderate clinical signs of dehydration, and 1 4 / of 42 patients showing but mild signs of dehydration. On the other hand 2.8 / of 76 cholera sufferers without clinical signs of dehydration had increased specific gravities of the blood (1 060—1 068) whereas according to El-Ramli the standard values in Egyptians ranged from 1 054 to 1.058

made in the form of a letter to the editor of the *Lancet* by O Shaughnessy (1831 32a) wherein he stated that

" (1) The blood drawn in the worst cases of the cholera is unchanged in its anatomical or globular structure.

" (2) It has *lost a large proportion of its water* 1000 parts of cholera serum having but the average of 860 *parts of water*

" (3) *It has lost also a great proportion of its Neutral saline ingredients.*

" (4) *Of the free alkali contained in healthy serum, not a particle is present in some cholera cases, and barely a trace in others*

" (5) Urea exists in the cases where suppression of urine has been a marked symptom.

" (6) *All the salts deficient in the blood, especially the carbonate of soda, are present in large quantities in the peculiar white dejected matters* "

The significance of these interesting findings, most of which were confirmed by further observations will be discussed in the following analysis of the physical and chemical alterations met with in the blood of cholera patients and occasionally also in that of victims to the disease

Physical changes in the blood of cholera patients

Early observations

To support his claim that in cholera " the essential and constant alteration of the blood consists in a decrease of the water content or what signifies the same in an increase of the solids " Liebermeister (1896) recorded the following early observations on the specific gravity and the fluid contents of the blood of the patients

Author	*Specific gravity of blood serum (normally about 1 028)*
Hermann (1832)	1 036 in a patient examined 4 hours before death
Wittstock (1832)	1.0385 — 1 0447
Thomson (1832)	1 0443 — 1 057
O'Shaughnessy (1832)	Usually about 1 040
Andrews (1832)	1 038 — 1.045
Schmidt (1850)	1 0286 — 1 0470 *

* Schmidt found the specific gravity of the whole defibrinated blood to vary in cholera patients from 1.0596 to 1 0711 as against 1 055 in normal men. In one instance the specific gravity of not defibrinated blood was 1 0728.

" According to the analyses of Schmidt " Liebermeister continued, " the water content of the whole blood in a healthy man was 78 87 %, in the male cholera patients examined by him 76.09—74.53—74 73 %, further in a healthy woman 82.46 %, in cholera-affected women 78 61—76.09 78 06 %. Wittstock obtained 26.5 % dry solids from the blood of a cholera patient so that there was 73.5 % of water Andrews found in four severe cases at the acme of the attack a water content of the whole blood amounting

"In order to estimate the actual degree of concentration of the blood in cholera, I have rapidly defibrinated the blood and centrifuged it in a haemocrite the percentage of corpuscules and serum being thus ascertained As the blood corpuscles are not lost (except to a very small extent, in the rare haemorrhagic cases) the amount of serum which has been drained from the blood can be estimated by a simple calculation."

Rogers claimed that the serum loss in the most severely affected cholera patients comprising those who succumbed in spite of treatment "amounted to no less than 64 per cent of the total" while those who could be saved by transfusion had shown a serum loss of 52% In mild cholera attacks followed by recovery even though no saline had been given, the serum loss averaged only 35% Hence, Rogers maintained,

"If we take the blood as one-thirteenth of the body weight and the volume of serum of these Bengali patients as 55 per cent, then in the fatal cases no less than 42 of the total 66 ounces of serum were on the average lost from the circulating blood alone, in addition to the great drain from all the tissues of the body Further in the second class of recoveries after transfusion, the loss averaged 34 ounces. Such great losses of fluid, accompanied by a corresponding degree of concentration of the blood, must necessarily embarrass the circulation, evidence of which is so clearly seen in the cold and blue extremities. In the severer cases the blood is so thick that it will not run into the capillary tube of a haemocrite without the aid of suction, so that its passage through the minute vessels of the pulmonary and systemic circulations must be very difficult."

Aron (1910) determining the water content of the blood in 8 cholera patients and also in the case of 6 cholera victims by drying the specimens at 99° to 100°C to a constant weight found that the percentage of solids varied in the patients blood from 21.5% to 28.5%, and in the dead bodies from 26.3% to 28 8%. Commenting upon these observations he stated that, while in four instances the blood of patients showed a normal or but slightly diminished water content in the four others the degree of water depletion corresponded to that met with in the blood of the dead bodies. It was noteworthy that in the case of the second group of patients the specimens had been obtained early in the disease Thus Aron s results were in agreement with the experiences of Schmidt (1850) who also found an increase in the blood solids in the early but not in the later stages of the disease

Once more using the haematocrit method Loh & Tai (1936) established that the average percentage of the blood corpuscle volume in 13 cholera patients examined before treatment was 63 1 becoming lowered after treatment to 48.2 The haematocrit figures ascertained in the case of 16 patients late in the disease varied considerably but were with a single exception surprisingly low

Reporting on the cell volume and the moisture content of the blood found in 17 cholera patients during the acute stage of the disease, Pasricha & Malik (1940) said in summary that (*a*) the cell volume varied from 36 3% to 69.3% with an average of 50.5% as against normal values ranging from 36% to 51% (*b*) the moisture content of the blood, determined according

Awny (1948)	While the blood specific gravity of his cholera patients varied from 1 056 to 1.078, in 18 out of 25 cases the plasma specific gravity was above 1 030
Ghanem & Mikhail (1949)	Examination of the specific gravities of the blood and the plasma according to the copper sulfate method in the case of 23 cholera patients revealed that (1) " Most of the cases with moderate or marked dehydration showed definite rise in the specific gravity of blood (13 out of 23) Because the figures on admission were higher than normal in only six of these cases due to associated anaemia, a single determination before treatment is considered unreliable as a sign or measure of the degree of blood concentration." (2) " All the cases with clinical dehydration showed higher specific gravity of plasma on admission than *normal (15 out of 23) only six of these showed high* specific gravity of the blood. This illustrates the value of this determination as an index and measure of the degree of blood concentration."
Saha & Das (1951)	Found the specific gravities of the blood and the plasma increased in practically all 49 cholera patients examined immediately after admission and before treatment was commenced.
Chakraborty (1954)	Determinations of the blood specific gravity in 31 dehydrated cholera patients showed values below 1 065 in 2 instances between 1.065 and 1 069 in 15 instances and between 1 070 and 1.072 in 14 instances.

The clinical significance of the findings recorded above and the methods used for determining the blood specific gravity will receive attention later in this chapter

Further observations on water depletion

As will be gathered from the summary of Liebermeister (1896) quoted above, a decrease in the fluid contents of the blood in cholera had already been noted by several of the earliest observers some of whom, e g., O Shaughnessy (1831 32b) spoke in this connexion of an increase of the " crassamentum " i e., the solid portion of the blood of cholera patients settling out on standing.

Investigations on this point have been continued by numerous subsequent workers, who were able to utilize improved methods of examination for thir purpose, particularly determinations made with the aid of the haematocrit. Though devised by Hedin as early as 1890 this method of comparing the volume of the blood corpuscles, " packed " by means of centrifugation, with that of the plasma, seems to have been used first in cholera work by Rogers, who stated in an article published in 1909 (a) that

The blood volume was determined according to a method described in detail by Chaudhuri and co-workers (1951a) In principle it consisted of (*a*) taking a 5 ml blood sample from an arm vein (*b*) then injecting through the needle, which had been left *in situ* an adult dose of 5 ml of a 0 7% Evans blue solution into the vein and (*c*) taking after 10 minutes a second blood sample (3 ml) from an arm vein on the opposite side Both these samples were centrifuged in haematocrit tubes and after readings had been taken the plasma was separated Next a known quantity of the dye solution was added to a measured quantity of undyed plasma from the first blood sample,

"so that this would serve as a standard against which the concentration of the dyed sample was compared. From the dilution of the dye injected plasma volume was determined, the estimation being done in a photo-electric colorimeter Blood volume was calculated from the plasma volume and relative haematocrit"

As described by the same authors (1951b) the available thiocyanate space was determined according to the technique of Crandall & Anderson (1934) To apply this, 10 ml of a 5% solution of sodium thiocyanate were injected into an arm vein after a 3 ml blood sample (hereafter designated as "sample A") had been withdrawn After one hour a second blood sample ("sample B") was taken from the opposite arm. The further procedure was as follows

"From both the samples serum is separated. In 0 9 c.c. of sample A is added 0.1 c.c of 1 100 dilution of stock thiocyanate solution In another tube 1 0 c.c. of the sample B is taken. To both these samples 1 0 c.c. of 20 per cent trichloro-acetic acid is slowly added and the protein is precipitated. Both these tubes are centrifuged for 20 minutes and the supernatant clear fluid is pipetted off To 1 0 c.c. of each clear aliquot, 8 c.c. of distilled water are added and finally one c.c of Reissner s reagent is added (Reissner s reagent—80 c.c. 10 per cent HNO_3 40 c.c. N $FeCl_3$ 40 c.c. distilled water) The buff colour developed is immediately matched in a photo-electric colorimeter

"The control sample A represents a dilution of the 10 c c. thiocyanate injected into 10,000 times and this dilution is compared with the unknown sample B Then, CNS space $= \frac{S}{R} \times 10{,}000$ where CNS space = available thiocyanate space in c.c., S = optical density of the standard and R = optical density of the unknown."

As they stated in their third communication (1951c)

"The interstitial fluid volume was calculated from the available thiocyanate space by deducting the plasma volume and 70 per cent of the r b.c. mass as calculated from the haematocrit."

The results obtained in 27 recently admitted patients with the methods described above were summarized by Chaudhuri and co-authors (1951c) in tabular form, showing for the two groups of these cases the mean values and standard deviations in ml per kg of body weight and per m² of body surface. These figures are reproduced below and the average values determined by these authors (1951a 1951b) in groups of healthy Indians have been added for the sake of comparison.

to a method described by Malik & Pasricha (1940) averaged 77 4 g per 100 ml of blood, as compared to normal values of 75 82 g per 100 ml of blood.

Taylor (1941) referring to a cholera enquiry under the auspices of the Indian Research Fund Association recorded that according to calculations made in the course of this work

" the total fluid loss including loss from blood plasma, intestinal fluid and intra-cellular fluid might be as high as 3.3 litres. Such loss might be equivalent to the total volume of the blood of the individual. It was estimated in the examination of cases that a blood concentration amounting to 140 per cent of the normal level was often present and that such concentration was a very dangerous level. Even at the equivalent of 125 per cent a dangerous condition was considered to exist."

Awny (1948) one of the several workers who made observations on the problems at present under review during the 1947 Egyptian cholera outbreak, stated that in his experience the volume of the packed red cells varied from 48% to 80%. Commenting upon these results as well as upon those obtained with the aid of other methods of blood examination which will receive attention elsewhere in this chapter he remarked that

" With very few exceptions the whole blood specific gravity the volume of packed red cells, the haemoglobin percentage, and the red cell count gave a fairly reasonable indication of the degree of haemoconcentration in any given case. In our experience, the indication derived from these data was also in close correlation with the clinical condition of patients. But they were of no prognostic significance whatsoever.

" The few exceptions referred to were cases who had high haematocrit readings together with low plasma specific gravity and plasma protein concentration, giving an erroneous finding for whole blood specific gravity As the main object of treatment at this stage is the restoration of a normal balance between the plasma and cellular elements the best thing to depend upon is a haemotocrit estimation. If this is not available, I think the next best would be a haemoglobin estimation or a red cell count. These are less elaborate than the specific gravity method, which necessitates much effort in the preparation of bottles which can be used for a few estimations only "

In strict contrast to this statement, Ghanem & Mikhail (1949) came to the conclusion that " the haematocrit reading has no value in gauging the degree of dehydration " in cholera patients. In support of this unusual contention the two workers pointed out that

" Only 11 cases showed higher haematocrit readings before than after treatment, five of these were in grade +++ [of clinical dehydration], six showed only mild degrees of dehydration (+) Moreover some cases with severe dehydration showed no rise in haematocrit reading."

For the purpose of studying the body fluid changes in cholera, Chaudhuri and co-workers (1951c) determined not only the haemoglobin content of the blood, the haematocrit values, and the protein and chloride content of the plasma in 27 recently admitted cholera patients but also the blood volume and the available thiocyanate space.

(c) *Interstitial fluid* While changes in the interstitial fluid were rather insignificant in the patients who afterwards recovered there was a 39% reduction of the mean normal value of the interstitial fluid in the sufferers who eventually succumbed This difference between the two groups of patients was most significant, because it was certain that a marked reduction of the interstitial fluid particularly if it remained long uncorrected was apt to produce irreversible changes in the environment of the body cells The authors insisted in this connexion that the immediate effect of hypertonic saline infusions was a withdrawal of fluid from the extracellular space into the circulation They admitted that such treatment was apt to lead to a rapid improvement of the circulation but maintained that

"in cases where extra-cellular fluid is grossly depleted, further reduction with hypertonic saline may be detrimental to the tissues themselves due to further change in their environment"

Further reference to the interesting observations and postulations summarized above will be made later in this chapter

Investigations similar to those just described were made also by Saha & Das (1951) As summarized in the *Tropical Diseases Bulletin* (1952) these authors

"estimate that for Indians of average weight and height the normal plasma volume will be 2.5 litres and the total extracellular fluid space will be 10 litres. On that basis and after making corrections for haemoconcentration and increase of the protein concentration of the plasma, the calculated average plasma volume of the series of cases examined is 1 6 litres.

"Estimation on four cases by the dye method gave an average plasma volume of 1 7 litres, representing a loss of 0 8 litres (32 per cent.) of the plasma fluid. By the thiocyanate method the average total extracellular fluid in the same cases was estimated at 7 7 litres. The total fluid loss is thus 2.3 litres of which 0.8 litre is from the plasma and 1.5 litres from the interstitial spaces."

It is important to note that the determinations made by Saha & Das as well as those of Chaudhuri and co-authors do not support the statements of some of the earlier observers who working with less reliable methods, were led to believe in truly amazing losses of fluids from the blood and the tissues Chaudhuri and his colleagues (1951c) while fully admitting the clinical significance of the plasma volume reduction in cholera, considered the most detrimental effect to be " the increased viscosity of blood with increased r b c mass which prevents maintenance of proper circulation in the body "

Observations on the erythrocytes

As far as can be ascertained Garrod (1849) was the first worker who pointed out that in cholera the " blood globules were found to be increased in amount " The unavailability of his original publication renders it impossible to establish what method of examination he used for this purpose.

Tests for	*Measure*	*Cholera patients*		*Healthy Indians*
		17 (recovered)	*10 (died)*	
Plasma volume	ml/kg	40.0±5 6	33.5±8.2	47 7
	ml/m²	1263 0±231 6	1074 0±207	1591.0
Blood volume	ml/kg	89 1±16.6	83.6±17.9	83 1
	ml/m²	2745.0±498	2795 0±614	2770.0
Thiocyanate space	ml/kg	233 4±24.5	165.8±29.3	236.3±30.8
	ml/m²	7008 6±825	5704 0±1,017	7401.0±817.3
Interstitial fluid	ml/kg	153 0±29.9	98.1±18.6	160 0±26.3
Red blood cell mass	ml/kg	45.5±12.4	46.0±15 1	
	ml/m²	1403 0±377	1559 0±477	
Total circulating chloride	mg/kg	229.8±53 7	215.8±61 6	

Repetition of these tests in 8 of the patients who recovered showed

" that plasma volume, interstitial fluid and circulating plasma chloride all increased with treatment, being actually higher than normal values in many of them, while the blood volume, r.b.c. mass, cell volume, haemoglobin and total plasma protein were reduced. Thus all the body-fluid and cellular changes were corrected with recovery and it seems some of them were over-corrected, probably due to excessive saline infusion."

Commenting upon their findings in the recently admitted patients, Chaudhuri and his colleagues made the following important statements

(*a*) *Plasma volume* The survival group of patients showed a 20 6% reduction of the mean normal value of the plasma volume per square metre as against a 32% reduction in those who did not survive. Though the latter degree of reduction was not necessarily the critical one it was most dangerous. However as in haemorrhage, the rapidity of the plasma loss was perhaps of greater importance than the actual amount of total reduction.

(*b*) *Blood volume* In contrast to the plasma volume the blood volume " was not reduced in any group as the packed-cell volume in all cases was much more increased than could be accounted for by the corresponding reduction in the plasma volume " In the opinion of the authors it was difficult to say whether this increase of the erythrocyte mass was apparent or real. They noted in this connexion that several workers had reported an unequal distribution of the erythrocytes, so that the blood drawn from larger vessels gave haematocrit values about 25% higher than the average body haematocrit figures. However Root and co-workers (1945) when determining the blood volume by other methods, had found little difference between the central arterial and the body haematocrit values. While it remained to be seen whether these findings were applicable in the case of cholera, it was possible that the increase of the red blood cell mass, found without exception in seriously affected patients, represented

" an attempt on the part of nature to compensate for the reduction of plasma volume, by pouring more cells into circulation from the large blood reservoir probably spleen "

(c) *Interstitial fluid* While changes in the interstitial fluid were rather insignificant in the patients who afterwards recovered there was a 39% reduction of the mean normal value of the interstitial fluid in the sufferers who eventually succumbed This difference between the two groups of patients was most significant because it was certain that a marked reduction of the interstitial fluid particularly if it remained long uncorrected was apt to produce irreversible changes in the environment of the body cells The authors insisted in this connexion that the immediate effect of hypertonic saline infusions was a withdrawal of fluid from the extracellular space into the circulation They admitted that such treatment was apt to lead to a rapid improvement of the circulation but maintained that

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According to Hayat (1948) this observation was confirmed by Hayem who in 1875 had already described methods of counting the blood corpuscles and in 1885 published together with Winter an article on the condition of the blood and the bile in cholera.

The wide spread of cholera in Europe in 1892 facilitated further investigations on this point Sticker (1912) mentioned in this respect that an increase of the red blood corpuscles to 6 million or even more had been noted in that year by workers in Hamburg. Biernacki (1895) referred to similar statements in the Russian literature particularly to observations made by Okladnych (1892) in the course of studies on the blood changes in cholera. Biernacki himself found in the algid stage of cholera a numerical increase in the red blood corpuscles which usually ranged from 6 5 to 7 5 million but was occasionally higher up to over 8 million in one instance. The few patients in the reaction stage to whom Biernacki referred usually showed about normal occasionally even subnormal values. Commenting upon his findings in the algid stage Biernacki maintained that though the numerical increase in the erythrocytes might be taken to indicate a state of blood concentration it could not be considered an exact yardstick for measuring that concentration.

Describing the blood findings in 23 cholera sufferers as well as in six patients with choleraic disease Rogers (1902) stated that

" In two of the [cholera] cases the number of red corpuscles rose to over 8 000,000 per cubic millimetre and both patients died, but one patient out of 3 cases in which they reached between 7 000 000 and 8,000 000 recovered, as did one patient (Case 1) in Table II [showing the findings in the sufferers from choleraic disease]. On the other hand, four patients out of five cases in which they numbered 5 000 000 and under also died, while out of 11 [?] cases in which they were between 5 000 000 and 7,000 000 seven patients recovered and only five died."

Rogers concluded therefore that there was no constant relationship between the degree of concentration of the blood as indicated by the number of erythrocytes and the death rate

Again referring to the subject under review Rogers recorded in 1921 that in the acute stage of cholera the number of erythrocytes was usually 6-8 million per ml but occasionally exceeded the latter figure Owing to the replacement of fluid there was usually a decline in the counts after the fourth day of illness, but not rarely the number of red blood corpuscles was found to be high in patients dying several days after admission with uraemic symptoms Though as stated above there was no constant relation between the number of erythrocytes and the death rate a very high count, since it indicated an excessive loss of fluid from the blood, was a bad sign.

Though Rosenthal (1914) dealing with the blood changes in cholera, gave details regarding the erythrocytes in one instance only these are of value in that they show that the number of red blood corpuscules, which showed an increase to 7 800 000 per ml on the first day of illness dropped

incessantly amounting to a little over 6 million on the 4th day to 5 852 000 on the 6th to 5 million on the 12th and finally to 3 764 000 on the 18th day after onset It may be conveniently added that there was also a continuous drop of the haemoglobin content of the blood from 115% on the first day to 73% on the 18th day of illness

Marcovici (1916) reporting on findings in 50 cholera patients stated that in the algid stage of severe cholera attacks the number of erythrocytes was usually increased to 7-8 million Though ascribing this phenomenon mainly to dehydration of the blood he drew attention to the fact that a moderate increase in the red blood corpuscles could also be met in slight cholera attacks Hence the possibility of an action of the cholera toxin on the haemapoietic system deserved attention

Wakamatsu Suzuki & Ando (1922) confirmed that there was a marked increase in the number of erythrocytes in the early stage of cholera the counts occasionally exceeding 8 million per millilitre The appearance of normoblasts sometimes observed indicated an unfavourable outcome

Findings similar to those of the above-quoted workers were recorded by Loh & Tai (1936) who stated that the average increase in the number of erythrocytes in the early stages of cholera was about 20%-25%, but that the values became practically normal after large amounts of saline had been infused

Awny (1948) while confirming that the number of erythrocytes in the blood of recently admitted cholera patients was invariably increased found that the highest value met did not exceed 7 5 million per ml As discussed above (see page 616) he maintained that, next to haematocrit estimations red cell counts or haemoglobin determinations represented expedient as well as dependable methods to gauge the degree of haemoconcentration in the patients and could thus serve as guides to adequate restoration of the fluid balance through saline administration

Recently De Bose & Mondal (1955) determined the number of erythrocytes present on admission (*a*) in 18 patients in whom the diagnosis of cholera was afterwards bacteriologically confirmed and (*b*) in a second group of 18 patients showing clinical features of the disease, but whose stools proved negative for *V cholerae* In the cholera group the average value of the red cell count was 5 83 million per ml (limits 4 1 million and 7 5 million) whereas in the vibrio-negative group the average value was 5 56 million per ml (limits 3 5 million and 6.5 million)

Haemoglobin determinations

Though an apparatus for the clinical estimation of haemoglobin was devised by Gowers in 1879 and a few years later Sahli (1886) introduced a universally known and still used method for the same purpose it would seem that advantage was not taken of these procedures in cholera work

earlier than during the 1892 outbreaks It is certain that in that year Oklad nych—as quoted by Biernacki (1895)—found that 24 cholera patients showed not only increases in the specific gravity of the blood and in the numbers of the red and white blood corpuscles but also an increase in the colour index (*Färbekraft*) of the blood

Rogers (1902) examining 23 cholera patients with the aid of Gowers method, found that, with few exceptions, the haemoglobin values were above—and usually well above—the low normal standard of 70 met with in the class of Indians among whom cholera was rampant The increased haemoglobin values varied from a minimum of 73 to a maximum of 130 according to Gowers standard

From a table in Rogers work on bowel diseases in the tropics (1921) it may be gathered that the average haemoglobin percentages determined at various stages of cholera compared thus with the average number of erythrocytes

Stage of the disease	*Haemoglobin percentage*	*Number of erythrocytes*
1st day	110	6 753 000
2nd day	104	6 106 000
3rd day	102	6 486 000
Later	94	5 745 000

Note Normal values were 70% for haemoglobin and 5 million for the erythrocyte count.

Using Sahli s method Marcovici (1916) as well as Loh & Tai (1936), found markedly increased haemoglobin values in the early stage of cholera. The first mentioned worker noted that these could reach 150% in severe and 120% in slight attacks of the disease In their 13 cholera patients, Loh & Tai found with one exception, haemoglobin percentages varying from 102 to 148 (average 122) on admission. After treatment these values dropped to an average of 90% The haemoglobin content in the blood of these patients thus exhibited changes parallel to those established by erythrocyte counts.

In order to determine the haemoglobin content of the blood of 17 cholera patients, Pasricha & Malik (1940) used a modification of the method of Newcomer (1919) and found values varying from 13 0 g to 26.2 g per 100 ml of blood with an average of 18.5 g, as against normal values of 13 g to 16 g. As the two workers added, the increases in the haemoglobin content observed by them were in direct correlation with increased haematocrit values.

Awny (1948) stated, without furnishing details, that the haemoglobin content of the blood of recently admitted cholera patients varied from 85% to 135%.

Chaudhuri and co-workers (1951c) found that, on admission, haemoglobin contents of the blood of 25 cholera patients varied from 14.3 g to 21.5 g per 100 ml, with values above 16 0 g in 17 instances It is important

to note in this connexion that the same authors (1951a) examining the blood of 50 healthy Indians, found, with few exceptions haemoglobin values well below this figure. In 8 of the cholera patients who could be examined before and after treatment, the mean haemoglobin content dropped from 18 7±6.8 g per 100 ml of blood to 11 9±1 4 g per 100 ml

De Bose & Mondal (1955) determined with a modification of Sahli s method the haemoglobin content in the blood of (*a*) 18 recently admitted patients suffering from true bacteriologically confirmed cholera (*b*) 18 patients who showed clinical features of the disease but whose faeces were negative for *V cholerae* and (*c*) an equally sized group of normal controls, and obtained the following results

	Haemoglobin percentages	
	Average	Limits
(*a*) Cholera group	111 66	95 and 130
(*b*) Patients with clinical cholera	109 72	70 and 130
(*c*) Normal controls	95 82	80 and 100

Erythrocyte sedimentation rate

The erythrocyte sedimentation rate in cholera was studied by De Monte & Gupta (1941) in view of the observations of Wolf (1924) who had found that the speed of sedimentation of the red blood corpuscles was accelerated in infants suffering from diarrhoea with toxic manifestations, but not in those affected by uncomplicated diarrhoea. Tests made with the aid of the apparatus of Westergren (1921) showed an increase of the sedimentation rate in 53 out of 79 cholera patients i e., in 67%. It was noted that the higher the specific gravity of their blood was at the time of the sedimentation tests, the greater was the number of instances in which no increase of the erythrocyte sedimentation rate was observable. Hence, as pointed out by Napier (1951)

"It would appear that there are two opposing influences, because it is mainly in the serious cases, in which the specific gravity of the blood is high, that the erythrocyte sedimentation rate is within normal limits. With clinical improvement it tends to rise."

Awny (1948) using Wintrobe s technique (see Wintrobe & Landsberg, 1935) found that

(*a*) in all 13 cholera patients examined before rehydration the erythrocyte sedimentation rate ranged from 0 45 mm/minute to 1 7 mm/minute (average 0 7 mm/minute) as against normal values of 0 1-0.3 mm/minute and

(*b*) in the 11 patients tested after complete rehydration the sedimentation rate varied from 0 6 to 1.5 mm/minute (average 1 0 mm/minute)

Noting that thus the figures obtained after treatment were higher than those before rehydration Awny considered it "possible that the higher viscosity of the blood before rehydration interfered with the sedimentation

earlier than during the 1892 outbreaks It is certain that in that year Okladnych—as quoted by Biernacki (1895)—found that 24 cholera patients showed not only increases in the specific gravity of the blood and in the numbers of the red and white blood corpuscles but also an increase in the colour index (*Färbekraft*) of the blood.

Rogers (1902) examining 23 cholera patients with the aid of Gowers method, found that, with few exceptions, the haemoglobin values were above—and usually well above—the low normal standard of 70 met within the class of Indians among whom cholera was rampant The increased haemoglobin values varied from a minimum of 73 to a maximum of 130 according to Gowers standard

From a table in Rogers work on bowel diseases in the tropics (1921) it may be gathered that the average haemoglobin percentages determined at various stages of cholera compared thus with the average number of erythrocytes

Stage of the disease	*Haemoglobin percentage*	*Number of erythrocytes*
1st day	110	6 753 000
2nd day	104	6 106 000
3rd day	102	6 486 000
Later	94	5 745 000

Note Normal values were 70 / for haemoglobin and 5 million for the erythrocyte count.

Using Sahli s method, Marcovici (1916) as well as Loh & Tai (1936) found markedly increased haemoglobin values in the early stage of cholera. The first mentioned worker noted that these could reach 150 / in severe and 120% in slight attacks of the disease In their 13 cholera patients, Loh & Tai found with one exception haemoglobin percentages varying from 102 to 148 (average 122) on admission. After treatment these values dropped to an average of 90%. The haemoglobin content in the blood of these patients thus exhibited changes parallel to those established by erythrocyte counts.

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of the worst cases, with a specially low percentage of chlorides, blood taken in glass tubes remained quite uncoagulated after several hours, and in one such case there were haemorrhagic stools, found *post mortem* to be dependent on extensive petechial haemorrhages in the caecum. After transfusion with hypertonic solutions the blood in such cases clotted firmly and gave a clear serum. In view however of the frequency of reduction of the clotting power of the blood in cholera, I now add 3 grams of calcium chloride to each pint of salt solution, and have seen no haemorrhagic stools in the few cases since treated."

Noting that a lessened coagulability of the blood had also been observed during the 1908-09 cholera outbreak in St. Petersburg Marcovici (1916) expressed the belief that this phenomenon was connected with atypical behaviour of the blood platelets which instead of being agglomerated in groups, as is normal were seen to lie singly in staneid smears of the blood from patients in the algid stage of the disease

Lahiri (1935) claimed in a preliminary communication that the coagulation time of the finger blood was shortened in most of the 116 cholera patients tested by him in this respect. A shift towards normal values (3-5 minutes) took place with clinical improvement. In Lahiri's opinion such examinations furnished more reliable clues regarding the general condition of the sufferers than specific gravity determinations.

In support of Lahiri's claim, Chatterjee (1946) stated that

"Procuring blood from a cholera patient is a comparatively difficult procedure owing to its high concentration and great liability to clot within the syringe."

It is also noteworthy that, as recently recorded by Chatterjee et al (1956) in a preliminary statement in 41 cholera patients studied by them there was a definite increase in the prothrombin time the value of which though sometimes remaining low even after the blood specific gravity had been restored to a normal level tended to become normal during convalescence A reduction of the prothrombin time was particularly noted in pregnant cholera patients As the authors added, "this seems not an unmixed evil as there is a greatly reduced post partum haemorrhage which seldom extends beyond the first day"

Blood viscosity

Though some workers such as Biernacki (1895) and as mentioned before Chaudhuri and colleagues (1951c) laid emphasis upon the role played by an increased blood viscosity in the causation of the general cholera syndrome, actual measurements of this phenomenon seem to have been made only by Wakamatsu and co-authors (1922) Using a method not specified in the review of their article by Takano Ohtsubo & Inouye (1926) they found that

"The viscosity of blood is markedly increased the highest observed was 22.8. In most cases the viscosity becomes normal in a week."

rate" He ascribed the discrepancy between his findings and the observations of the Indian workers quoted above to the fact that—as was essential—he had corrected his results with the aid of those obtained through haematocrit readings.

Erythrocyte fragility

In the paper just quoted Awny (1948) also stated that in 14 cholera patients

" fragility of the red cells was estimated by the Sanford technique [1] (diminishing dilutions of sodium chloride solution) Taking the dilution of 0 45 % as the critical concentration at which normal red cells reveal initial haemolysis, we found that 13 out of our 14 patients showed increased fragility of their red cells during the acute stage of the disease. In six patients serum was obtained for two purposes First, to determine the red cell fragility using the Wiseman technique [1] (increasing dilutions of the serum with distilled water) second, to determine the serum icterus index. This was performed during the acute stage and after complete recovery During the acute stage the results showed a definitely increased fragility of the red cells which later became normal during convalescence."

For the convenience of record it is added that in these six cholera patients the icterus index (determined presumably through comparisons with standard solutions of potassium bichromate) was high (between 12 and 23 units) during the acute stage of illness but dropped to less than 8 units during convalescence

Coagulability of the blood

Discussing the blood changes in cholera Sticker (1912) drew attention to observations of Orton (1820) and other pioneer workers, according to which the blood in cholera showed little tendency to coagulate Even earlier than that the English medical men working during the 1817 outbreak in India had noted that great care had to be exerted when resorting to venesection in the early stages of cholera, because it was difficult to stop the flow of blood initiated in this manner Hand in hand with clinical improvement, the coagulability of the blood increased and the separation of the fluid from the solid part of the blood, which was formerly absent, took place in a typical manner

Again referring to this subject in modern times, Rogers (1909b) stated the following

" A few observations on the clotting power of the blood have been made by Sir Almroth Wright's method (1893) The results were very variable, the time being normal in some, slightly reduced in most, and very markedly in a few It is noteworthy that in several

[1] The method described i[n] the handbooks on laboratory methods under the name of Sanford's test was originally introduced by Gaffen & Sanford (1919). For Wiseman's technique, see Wiseman & Beerbaum (1932).

in slightly affected cholera patients as well but he also referred to one slightly affected sufferer who showed a leucopenia instead

From a table summarizing Biernacki s findings in 8 severely affected patients examined either during the algid stage or later in the disease, the following data can be culled

	Algid stage	*Reaction stage*	*(Normal values)*
Number of leucocytes	26 562 57 031	17 968 35 781	(7 500)
Number of erythrocytes	6 000 000-7 662 000	3 193 750-5 987 500	(5 000 000)
Proportion of leucocytes to erythrocytes	1 111 to 1 288	1 136 to 1 333	(1 666)

Note In a 9th patient who could be examined twice, the number of leucocytes per ml was 27 500 (i.e., 1 234 erythrocytes) during the algid stage, and 14 062 (i.e., 1 352 erythrocytes) during the stage of reaction.

Differential counts convinced Biernacki of the preponderant presence of neutrophil polynuclear leucocytes in the blood of cholera patients (82.5% 95 4% in the 4 patients for whom he gave details) Eosinophil cells were never seen

Dealing with the prognostic significance of the results of leucocyte counts, Biernacki stressed that all patients who showed an extremely high leucocytosis (40 000 or more per ml) in the algid stage rapidly succumbed However a high leucocytosis was not invariably present in the sufferers who succumbed to the disease Biernacki added that

"an increase in the number of the leucocytes in the reaction stage was also correlated with a serious character of the illness but it was not regular enough to serve *per se* as a diagnostic and prognostic sign" [Trans.]

Still, the number of leucocytes decreased more rapidly in the patients who soon began to void urine than in those with signs of uraemia or cholera typhoid

Recording the findings he made in 23 cholera patients as well as in 6 individuals suffering from non-choleraic diarrhoea, Rogers (1902) stated the following

"It has long been known that leucocytosis is a well-marked condition in Asiatic cholera, while it is said to be most marked in the severest and most fatal cases. These statements are borne out by my observations, leucytosis having been present in every case which was examined in the earlier stages—that is, within the first two days of the disease. In nine cases it was present in comparatively slight degree less than 20,000 leucocytes per cubic millimetre and five of these patients recovered. On the other hand, out of 14 cases in which this number was exceeded 11 patients died and only three recovered, these three, however including two out of the three cases in which over 40 000 leucocytes were found per cubic millimeter A high degree of leucocytosis is, then, a bad prognostic sign and a slight degree a good one, yet a very high degree is not incompatible with recovery The constancy of the occurrence of leucocytosis in cholera is of importance in distinguishing it from some forms of acute diarrhoea in which it is absent It is not, however of absolute value, as leucocytosis occurs in ptomaine poisoning and *sometimes*

It would be certainly desirable to obtain further information regarding the viscosity as well as the coagulability of the blood in cholera.

Observations on the blood platelets

As far as can be ascertained observations on the blood platelets in cholera were made first by Chistovich (Tschistowitsch 1909) during the 1908-09 outbreak in St. Petersburg, who considered a diminution of the number of the platelets as characteristic of this disease Marcovici (1916) maintained that Chistovich must have made his observations on cholera patients in the later stages of illness because in his experience the number of the blood platelets increased considerably (up to 1 million per ml) in the algid stage whereas in convalescents a disintegration and a marked numerical decrease in the platelets became noticeable However Wakamatsu and co-authors (1922) recorded once more that in cholera the blood platelets " at first tend to decrease but they increase again after recovery of the patient "

This discrepancy of opinion is rather distressing.

Observations on the leucocytes

Try as the present writer might, the information he was able to procure regarding early observations on the leucocytes in cholera is incomplete Griesinger (1857) stated in his treatise on infectious diseases, without giving a reference that, as in many other diseases so also in cholera attacks, the number of the " colourless " blood corpuscles was increased, but that " no certain explanation could be given for this phenomenon " Possibly Griesinger referred in this connexion to findings of Virchow who according to some later compilers, established the presence of a leucocytosis in cholera, presumably afterwards recording this fact in his collected papers on public health subjects (1879) As Macleod (1910) added, identical observations were later published by Lewis & Cunningham, evidently in one of the *Reports of the Sanitary Commissioner with the Government of India* (date not specified) According to Hayat (1948) the presence of leucocytosis in cholera was also referred to quite early by Hayem (see Hayem & Winter 1885)

Towards the end of the 19th century further observations on this point were recorded by Okladnych (1892) and by Biernacki (1895) According to the latter author Okladnych observed a high degree of leucocytosis in all the 24 cholera patients examined by him.

Biernacki seems to have been the first worker to establish that leucocytosis was marked not only in the algid stage of cholera gravis (during which presumably the early observers made their examinations) but also in the later stages of the disease He found instances of *exquisite Leukocitose*

"there was a correlation between the number of leucocytes and toxic symptoms and patients with over 40 000 leucocytes invariably showed a serious clinical picture" [Trans.]

However two patients with 21 600 and 18 400 leucocytes respectively per ml rapidly recovered

Summarizing the results of previous observations on the blood in cholera, Benzler (1916) took it for granted that, besides an *apparent* increase in the number of the white as well as of the red blood corpuscles as a consequence of the concentration of the peripheral blood there was also a *real* numerical increase in the leucocytes due presumably to an action of the cholera toxin While it was well established that the neutrophil polynuclear cells prepon derated in this increase in the leucocyte counts In Benzler s opinion less satisfactory information was available in regard to the role of the large mononuclear cells and the lymphocytes For this particular reason as well as on general grounds, he therefore made a careful study of more than 11 000 blood cells in 30 stained blood smears prepared at various stages of the disease from 20 cholera patients and recorded the results according to the well known system of Schilling (1911) As shown by this study the behaviour of the leucocytes in the successive stages of cholera was as follows

(1) *Prodromal stage* While, apart from a possible increase in the large mononuclear cells, there were, generally speaking, no abnormal findings, the presence of prodromal diarrhoea led to lymphopenia and mononucleosis.

(2) At the *onset of the disease* the number of the lymphocytes showed a marked drop moreover a mononucleosis associated to some extent with the presence of irritation forms, and a slight hyperleucocytosis appeared.

(3) *Algid stage* There was a most marked lymphopenia, which sometimes almost assumed an aplastic character The presence of a marked mononucleosis was due to the massive appearance (*Ausschüttung*) of atypical forms.

(4) In the *stage of reaction* there was a gradual increase in the number of the lymphocytes and a numerical decrease in the large monocytes. The prognosis remained doubtful if in spite of a numerical increase in the lymphocytes, the number of the large mononuclear cells remained comparatively high.

(5) *Convalescence* Besides a marked lymphocytosis a reappearance of irritation forms and a numerical increase in the eosinophils could be noted. There was also a slight neutropenia and a disappearance of the atypical large mononuclear cells.

Since, as stated, for instance by Naegeli (1923) a lymphocytosis often appears after the administration of bacterial vaccines, it is tempting to associate the numerical increase in the lymphocytes which becomes manifest in cholera patients late in the disease with the development of immunity to the infection

Examining the blood of 50 cholera patients in the usual manner Mar covici (1916) made the following noteworthy findings

(1) During the algid stage of the disease the number of leucocytes varied from 8000 to almost 40 000 a high degree of leucocytosis rendering

in cases of acute dysentery beginning with severe diarrhoea and it is just these which for a time present the greatest difficulty in diagnosis."

Making differential counts, Rogers found that

(1) The percentage of polynuclear leucocytes was increased to about 80% as against a normal value of 68%.

(2) The number of lymphocytes, which was normally 25%, was markedly decreased, being under 10% in 13 out of 17 cases examined on the first or second day of the disease

(3) On the other hand, the number of the large mononuclear cells was usually increased above the normal percentage (6%) as well as absolutely

(4) As can be gathered from the table given in Rogers article, the percentage of eosinophils was with some exceptions well below the normal of 1%

Rogers maintained that the decrease in the lymphocytes and increase in the large mononuclear cells which were always found were of diagnostic value since corresponding changes were absent in three patients with non choleraic diarrhoea, even though two of them showed the presence of leucocytosis.

He also postulated that these findings were of prognostic value since

(a) Out of 16 cholera patients in whom less than 10% of lymphocytes had been found, not less than 12 succumbed, whereas out of 7 sufferers with more than 10% lymphocytes, four recovered and

(b) Out of 6 patients in whom under 10% of large mononuclear cells were found, four recovered, while out of 15 showing more than 10% of these cells, 12 died.

Further important observations regarding the behaviour of the leucocytes in cholera may be summarized as follows

Rosenthal (1914) stated that

(a) In all 30 cholera patients examined by him there was a marked, often even an extremely high leucocytosis (several times over 40 000 once 76 400 per ml) which was thus quite out of proportion to the blood concentration manifested by an increase in the erythrocytes and the haemoglobin values, and which though decreasing from day to day persisted to a lesser degree "fairly long" (*recht lange*)

(b) Throughout the illness the large mononuclear cells were far more numerous than, or at least as numerous as the lymphocytes.

(c) The eosinophils were rare or often even altogether absent early in the disease but—to judge from apparently few observations—reappeared towards the end of the first week of illness and reached 10% in the case of a patient with a specific skin rash examined on the 10th day after onset.

While denying that leucocyte counts of 40 000-50 000 or more per ml rendered the prognosis invariably infaust, Rosenthal admitted that

are decreased from the beginning, and if the diminution is progressive the prognosis is poor. The large mononuclear cells are slightly decreased during the acute stage but the decrease is within physiological limits. They increase with the recovery in number of other leucocytes. Eosinophile cells usually disappear during the acute stage but in a few cases which died with uraemia there were eosinophile cells just before death."

Reporting in detail on the blood examination of 13 cholera patients Loh & Tai (1936) recorded the constant presence of a leucocytosis the number of white blood corpuscles before saline infusions were given averaging 22 280 cells per ml and decreasing after treatment to an average of 17 700 cells per ml. The two workers ascribed the persistence of a high white cell count to a leucocytic reaction to the infection

Differential counts made by Loh & Tai yielded no significant results the average percentages of the various types of white blood cells before and after saline treatment remaining practically unchanged. Quite possibly the second determinations had been made soon after rehydration

Awny (1948) reporting on haematological studies during the 1947 Egyptian cholera epidemic, stated that

"In most of the cases, the white blood corpuscles showed a definite increase which could not be explained solely on the basis of haemoconcentration. Figures between 20,000 and 30,000 per cubic mm. were quite common. In one case the count was 43 000 per cubic mm. If we compare this increase with the rise in red cell counts we come to the conclusion that there was a definite leucocytosis in addition to the increase due to haemoconcentration.

"This leucocytic reaction was found to involve mainly the neutrophile polymorphonuclears. There was a definite lymphopenia and a disappearance of the eosinophils. There was also some left shift in the polymorphs. No abnormal cells were observed in the circulation.

"After subsidence of the acute stage of vomiting and diarrhoea, and correction of the dehydration, the total leucocytic count was still high in most of the cases, ranging from 9000 to 19 000 with an average of 11 14 000 per cubic mm. Differential counts showed a persistence of the lymphopenia, a slight increase in monocytes, a definite elevation of the neutrophilic polymorphs, and a reappearance of the eosinophils and basophils.

"During convalescence, the total counts were still high ranging from 7,000 to 14 000 per cubic mm. This stage was characterised by an elevation of the percentage of eosinophils in the differential counts. This elevation might have been due to parasitic infestation of the patients or to antibody formation of which allergy is a manifestation. This point ought to be further investigated."

The results of total leucocyte counts obtained by De and co-workers (1955) in their three groups of 18 individuals each (see page 623) were as follows

	Number of leucocytes per ml average	*limits*	*Average ratio of leucocytes (in thousands per ml) to erythrocyte (in millions per ml)*
(a) Cholera group	12 638	4 500 and 25 000	2.17
(b) Patients with clinical cholera	10 193	3 500 and 16 000	1 83
(c) Normal controls	6 806	4 400 and 9 500	1 40

the prognosis unfavourable. The usually marked leucocytosis was of a neutrophil type the lymphocytes and large mononuclear cells being scanty (up to a maximum of 3% and 8% respectively) and the eosinophils absent or rare

(2) With the onset of convalescence in the second week of illness, owing perhaps to an action of the cholera toxin on the lymphatic system the number of white blood corpuscles was still high (10 000-15 000 per ml) However there was a marked decrease in the neutrophil leucocytes with a corresponding preponderance of the mononuclear cells especially the lymphocytes and macrolymphocytes (irritation forms) The number of eosinophils increased up to 6% A disintegration of polynuclear leucocytes as well as of the blood platelets seemed to be characteristic of this stage.

Banerjee (1921) using the leucocyte classification system of Arneth (1904) for a study of 51 cholera patients, came to the conclusion that (*a*) in this disease there was a well marked "shift to the left" the average index being 71 9 as against 52.55 in healthy Bengalis and (*b*) Arneth s index was of use in determining the severity of cholera attacks the average figure in the sufferers who afterwards succumbed being 81.30

Reporting in a second paper on a general study of the leucocytic picture in 100 healthy Bengalis and the 51 above mentioned cholera patients Banerjee (1922) gave the following summary of his findings

"1 Differential counts of average healthy Bengalees show slight increase in Large Mononuclears and Eosinophiles, the average being —

Poly	*Large Mono.*	*Small Mono*	*Eosino.*
67.22	9 67	18.42	4 69

"2. Total count of healthy Bengalees is on an average — 7698

"3 Differential count of cholera cases shows marked Mononuclears increase proportional decrease of Lymphocytes, which is peculiar to cholera and is not found in any other disease. The average being,—

Poly	*Large Mono*	*Small Mono*	*Eosino.*
72.55	16 83	10 03	0.59

"4 Total leucocyte count in Cholera cases shows marked leucocytosis more specially in fatal cases, but very high leucocytosis is not incompatible with recovery Majority of cases showing count between 10 000 to 30,000. This leucocytosis is both absolute and relative, and has got no relation to the concentration of the blood."

Takano and co-authors (1926) summarizing the findings made in regard to the behaviour of the leucocytes in cholera by Wakamatsu and others (1922) stated that

"The number of leucocytes is highest at the uraemic period, and most of the cases who had over 20 000 died. The highest count was 38 000. In the final stages of the disease, the leucocyte count is low The ratio of red to white cells is always less than normal because of the increase of the leucocytes. The increase of the polymorpho-leucocytes at first is slight, but with the onset of the intoxication symptoms they gradually increase, and finally myelocytes may begin to appear and justify a bad prognosis The lymphocytes

manifest in this disease For even though De and co-authors failed to obtain positive bacteriological results in their second group of cases it is quite likely that these patients who showed signs typical of severe cholera attacks, actually suffered from this disease Moreover it stands to reason that other organisms, which are capable of producing such a syndrome possess like *V cholerae* pathogenetically active endotoxins

An additional problem of considerable interest is whether the physical changes of the blood in cholera dealt with earlier in the present chapter are exclusively the result of a concentration of the peripheral blood As noted before (see page 621) Marcovici (1916) finding that a moderate numerical increase in the erythrocytes was also met in patients with slight attacks of cholera which did not result in marked dehydration postulated that an action of the *V cholerae* endotoxin was to some extent responsible for this hyperglobulia.

Support for this contention, which certainly deserves serious consideration, may be found in experimental observations made long ago by Grawitz (1893) This worker established that (*a*) intravenous injection of rabbits with old unsterilized or sterilized cholera broth cultures led to a concentration of the blood of the animals manifested by an increase in the specific gravity whereas (*b*) no such effect could be produced with young *V cholerae* growths. Ascribing, therefore his results not to an action of the cholera vibrios themselves but to that of their metabolic products Grawitz made the following interesting comment

"*It is most suggestive to think of the haemoconcentration which takes place in human* cholera and which is generally considered to be merely a consequence of the watery excretions in the intestine. However it seems well worth considering that besides this factor an influence is exerted as well in the human body by the proven lymphagogue [*lymph treibende*] action of the metabolic products of the cholera bacteria, and that this action *perhaps even plays a very important role, if one takes into account the massive multipli*cation of these bacilli in the intestine of the patient, in comparison to which the small quantities of my artificial growths are well nigh infinitesimal and if one further considers how favourable conditions exist for a resorption of the metabolic products at the time of their formation in the intestine." [Trans.]

It might be well to repeat these experiments, preferably using other species of laboratory animals particularly rats in addition to rabbits

For the convenience of record, reference is made at the present juncture to a recent study of the sternal marrow picture in cholera made by Ata (1954) Examining 18 cholera patients (7 of them twice at an interval of one week) this worker found that the total cell count in the sternal bone marrow varied from 9000 to 336 000 per ml however in only 6 instances were the counts outside the normal range (20 000-100 000 cells per ml) The number of myeloblasts and myelocytes was found to be invariably below normal, while the number of lymphocytes showed an increase in 14 of the patients and that of the plasma cells in two instances It is note

While pointing out that haemoconcentration was partly responsible for the increase in the number of leucocytes noted in the two groups of patients, De and his colleagues justly maintained that

"a rise in the white cell/red cell ratio in both groups of patients, indicating a disproportionate increase of the whites, suggests the occurrence of a real leucocytosis"

Differential leucocyte counts gave the following results

	Percentages		
	neutrophil polymorphonuclears	*eosinophils*	*lymphocytes*
(*a*) Cholera group	82.5	0.83	16.30
(*b*) Patients with clinical cholera	82.2	1 17	17.55
(*c*) Normal controls	58 0	5.39	35 78

Thus there was in the cholera group and somewhat less markedly in the group of patients with clinical signs of the disease a neutrophilia associated with an eosinophilia and a lymphopenia. In regard to the last, however it is noteworthy that these authors found no reduction in the number of lymphocytes in two of their cholera patients who to judge from the unusually large amounts of saline needed for their rehydration suffered from particularly severe attacks But, they added a fatal issue was noted in a third sufferer showing a lymphopenia associated with an unusually large number of eosinophils.

Though by no means invariably in agreement in regard to details, especially as far as the results of differential counts are concerned, practically all recent workers making haematological studies in cholera patients were agreed as to the presence of a leucocytosis in this disease which (*a*) was disproportionally higher than the numerical increase in the red blood cells, and (*b*) in contrast to the latter continued to be manifest to a considerable degree in the latter stages of the disease even after the number of erythrocytes had become normal

While therefore unanimous in stating that it was impossible to ascribe this leucocytosis merely to a haemoconcentration the various workers reached no general agreement regarding the nature of the additional factor at work. A few e g. Loh & Tai (see above) spoke somewhat vaguely in this respect of a "leucocytic reaction to the infection" The generally accepted opinion ascribes the presence of a disproportionally high and persistent leucocytosis in cholera to an action of the *V cholerae* endotoxin on the haematopoietic system. However De and co-authors, commenting upon their observations on the leucocytes and also on the sugar content in the blood of their two groups of patients (see below) suggested that

"Identical blood changes in both groups of cases point to the non-specific stimulation of the pituitary-adrenal axis by the associated dehydration, shock and anoxia rather than by the products of *V cholerae*"

It would seem, nevertheless that it would be rash to discount the role of the cholera endotoxin in the production of the leucocytosis which becomes

"would be associated with an increase of salts in the circulating blood and this condition might act as a conservative process by producing osmotic currents carrying fluid into the *blood rather than from it*"

The observation often made that intravenous infusion of large amounts of normal saline though leading to a temporary improvement in the general condition of the patients was apt to be followed by renewed evacuations from the bowel seemed to support the above assumption because as a result of this treatment the supposedly high salt concentration of the blood would become lowered

However making a series *of observations with the aid of silver nitrate* titrations Rogers found that the chloride content of the blood of his patients was below the normal standard which as he assumed on account of the investigations of McCay et al (1907) was higher in Indians than in Europeans reaching almost 1%. He recorded in this connexion average values of 0 79% in 7 patients who afterwards succumbed to the disease and of 0 9% in 12 patients who could be saved through subsequent treatment *with hypertonic saline An average chloride content of 0 92% was found in* 5 sufferers from mild cholera attacks who did not require saline infusions

Hence as Rogers still maintained in 1952,

"The salts are also lost from the blood [of cholera patients] in large amounts this loss may even be greater than that of the fluid so that the percentage of chlorides actually falls below the normal of 0.85 per cent., and in extreme cases haemolysis may take place in the circulation"

It is important to note that on this occasion as well as previously in his 1921 book Rogers considered a chloride content of the blood of 0 85% as the normal standard. There can be no doubt that the figure of nearly 1% for Indians had been arrived at by McCay et al (1907) with an inadequate technique

Aron (1910) determined the chloride content of the blood of 9 cholera patients and of 5 cholera victims by incinerating the specimens after admixture of sodium carbonate at a low temperature and subsequently resorting to precipitation with silver nitrate the amount of silver chloride present then being determined gravimetrically or by titration

As can be gathered from Aron s tables, examination of these blood samples as well of serum samples of 4 cholera patients showed in nearly all instances a decreased chloride content—a result which was in accord with the experiences of Schmidt (1850) and Rogers (1909a) Turning attention to the question whether as postulated by the latter worker owing to a disproportionally large salt loss the blood of cholera patients became hypotonic Aron made the following interesting statement

"We have seen that the blood of cholera patients loses water An *isotonic* blood, with a lower content of water should have a lower content of salts (chlorides) The decrease in salts (chlorides) in itself does not prove that the blood has become *hypotonic* The quantity of salts is often reduced in relation to the amount of total solids, but this cal-

worthy that the increase in the lymphocytes in the bone marrow was as a rule not accompanied by a corresponding rise in their number in the peripheral blood. In all but one of the patients examined the number of normoblasts in the bone marrow was lower than normal The myelogram as well as the counts in the peripheral blood were found to have become normal in convalescence.

Chemical changes in the blood of cholera patients

Early investigations

Though, as quoted above (see page 612) O Shaughnessy (1831 32a), investigating the chemical properties of the blood in cholera, reached conclusions which have been endorsed by modern observations, some of the other early workers in this field were not in accord with his findings, so that this problem soon became the subject of much debate. While it would serve no useful purpose to deal with this controversy in a detailed manner attention is due to the observations of Schmidt (1850) which indeed may be said to have ushered in the modern phase of the problem at present under review

As tabulated by Biernacki (1895) and again by Aron (1910) the chemical findings made by Schmidt in the blood of five cholera patients, examined at varying lengths of time after onset, were as follows

Case	*Clinical data*		*Percentages of*					
	hours after onset	*outcome*	*chlorides* (Cl)	*phosphorus pentoxide* (P_2O_5)	*potassium oxide* (K_2O)	*sodium oxide* (Na_2O)	*phosphate of calcium*	*phosphate of magnesium*
1	3	Died	0.228	0 0314	0.194	0 158	0.032	0 096
2	9	Died	0.222	0 0746	0.203	0 149	0.046	0.047
3	12	Died	0.259	0.0887	0.225	0 140	0 074	
4	18	Cured	0.221	0 0612	0.166	0 172	—	
5	36	Died	0.195	0.0809	0.184	0 111	0 086	
Normal values			0.262	0 0766	0.173	0.190	0 039	

Note Examining the chloride contents of the serum in four of these patients, Schmidt found percentages of 0 354 (case 1) 0.296 (case 3) 0.305 (case 4) and 0.314 (case 5)

As far as these figures go—the adequacy of Schmidt s normal standards has been doubted—they indicate (*a*) a marked decrease of the sodium percentages in all, and of the chloride percentages in most of the specimens, and (*b*) increases in the potassium as well as in the phosphorus or phosphate contents of the blood of cholera patients

Owing to their fundamental importance the investigations of Rogers (1909a, 1909b) and of Aron (1910) though following those of Schmidt after a long interval of time, also deserve attention at the present juncture.

As pointed out by Rogers (1909a) it was to be expected *a priori* that the abundant loss of fluids from the blood of cholera patients

Pasricha & Malik (1940) using a micro-method described in detail by Malik & Pasricha (1940), made similar observations on 10 still untreated patients in the collapse stage of cholera and maintained that, though a diminution of the NaCl concentration was noticeable in the blood and in the plasma of the sufferers, this reduction was not marked the average sodium chloride content in the blood was 435 mg per 100 ml as against normal limits of 450-530 mg, whereas the corresponding figure for the plasma of the patients was 586 mg as against normal values of 560-620 mg per 100 ml

In marked contrast to these findings, Banerjee (1941 a) determining the sodium chloride content in the blood as well as in the stools of 20 cholera patients, mostly from day to day emphasized that, even though these sufferers had been amply treated with saline infusions (average NaCl intake 25 g per day for the first 2 3 days after admission) the blood chlorides never rose above 495 mg per 100 ml, and in a large number of the patients varied from only 350 mg to 450 mg Still lower values were found in the blood samples taken before treatment had been initiated

Concluding, therefore, that " in cholera, hypochloraemia is invariably present in all cases " Banerjee added the following important statement

" As a threshold substance NaCl with a threshold value of 560 mg. to 570 mg. per 100 c c. of blood is re-absorbed through the [kidney] tubules back to the circulation in order to maintain its equilibrium in the blood. In cholera, as the plasma chloride concentration is lowered to the limit of the threshold value, a decrease in urinary chloride occurs The diminution in the plasma chloride concentration produces a great altera tion in the distribution of the electrolytes and in the acid-base balance This constitutes one of the most significant metabolic features in conditions producing hypochloraemia and particularly in cholera."

Studying the chemical changes of the blood in 105 cholera patients Chatterjee & Sarkar (1941) for determination of the chlorides resorted to the method of Van Slyke (1923) Though as will be further discussed below it appeared that the loss of sodium from the blood of these sufferers was more considerable the two workers nevertheless invariably noted a diminished chloride content of their specimens Moreover, they stressed with great reason that, when assessing the significance of such alterations or of any other chemical alterations of the blood in cholera, it was essential to consider the results in relation to the degree of blood concentration Thus, the apparently slight lowering of the chloride content of the blood in severely cholera affected and consequently much dehydrated patients referred to by Rogers (1911 1921) actually indicated a marked loss of chlorides parallel with that of fluid from the blood

Further observations on this problem made during the 1947 cholera outbreak in Egypt showed the following

Hafez (1947) estimating the blood chlorides in 24 patients, found values below the normal standard in eight instances only

culation is apt to convey a wrong idea with regard to the tonicity of the fluid. The quantity of salts should be compared in relation to the quantity of water and then it should be determined whether the loss in salts is proportional to the loss in water or greater or less. If we do this, we will see that in the samples obtained during the first three days of the disease we can scarcely speak of a greater loss in the salts than would correspond to that of water Of course, in the later stage of the disease, in Schmidt's analyses as well as in my own, the water content of the blood is almost normal, while that of salts, estimated as chlorides, is below that point. At this time we really would have a hypotonic blood"

Making a few additional analyses of the blood samples obtained from cholera victims, Aron found a marked decrease in the sodium content as well as slight increases of potassium and phosphorus—findings in agreement with the more numerous observations of Schmidt. As Aron added, the increase in potassium

"is readily explained if we consider that cholera blood contains a higher percentage of red blood cells than normal"

The rise in the percentage of erythrocytes as well as an increased protein content seemed to account for the higher phosphorus content of the blood in cholera.

Further observations on the electrolyte content of the blood in cholera

(1) Chlorides Shorten (1918) estimating the "chlorine" content in the serum of 11 patients in various stages of cholera as sodium chloride according to a method described in detail in the first part of his article, stated that

"The chlorine content would appear to be slightly increased early in the disease before treatment is begun but taking into account the great loss of fluids from the blood, the loss of chlorine to the body must be considerable... From the figures it will be seen that there is a general tendency to a fall in the chlorine content as the case progresses but it is remarkable how closely the figures in cases treated by the hypertonic method approximate the normal."

Again dealing with the problem under review Liu, Wang & Fan (1933b) recorded that in 13 cholera patients who could be examined during the acute stage of the disease usually before treatment had been commenced, the chloride concentration of the blood was reduced to an average of 92.2 milli-equivalents per litre Re-examinations of the 12 surviving sufferers during convalescence showed that the chloride content of their blood had risen to an average of 99 2 milli-equivalents per litre

Making further observations on 13 still untreated cholera patients, Loh & Tai (1936) found that the sodium chloride content of the blood of these sufferers, determined by Whitehorn's method (1921) was but slightly decreased (average value 503 mg NaCl per 100 ml of blood) Saline infusions brought the salt content of their blood to an average value slightly above normal (575 mg NaCl per 100 ml of blood)

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Hafez (1947) estimating the blood chlorides in 24 patients, found values below the normal standard in eight instances only

Noting that the normal chloride content of the blood in Egyptians varied from 479 to 500 mg% El Ramli (1948) stated that

"In the 25 cases examined during the acute stage, the blood chlorides were found to vary from 432 561 mg./ but mostly around 470 or slightly over except in one fatal case which started at 421 and ended with 374"

Safwat & Adham (1948a) maintained that

"Hypochloraemia was not found to be a marked feature in the 32 cases examined. We had only 4 cases with figures definitely below normal levels. Moreover on fluid treatment with normal saline, chlorides quickly rose and remained at the new level"

However details furnished regarding these investigations in a second paper by Safwat & Adham (1948b) do not quite bear out their general statement just quoted As noted in the second article the lowest chloride figures in the blood of the 32 patients ranged from 450 mg% to 470 mg%, but as a rule values varying from 470 mg% to 500 mg% were obtained. Since the two workers maintained in contrast to El Ramli that the "normal figure in Egyptians is estimated to run between 479 and 600 mg%" it would appear that usually at least a hypochloraemia was initially present in their patients which, in relation to their dehydrated condition cannot be considered "very slight"

It is noteworthy however that the presence of hypochloraemia in cholera was again denied by Ghanem & Mikhail (1949) who stated in this respect that

"Estimations of blood chloride [with the aid of direct titration] were carried out before and after treatment in 16 cases It was increased above the normal in all cases, the figures ranged from 520 to 710 mg. per cent. (the normal is 450 to 520) In seven cases, it showed higher figures on admission than after treatment, and in eight cases the blood chlorides were lower on admission than after treatment."

The two workers admitted, however that

"this estimation of the plasma chlorides is fallacious as an indicator of the total loss of chloride in mixed depletions because of the tendency to hypertonicity in the diminished extracellular fluid"

Similarly as quoted in the *Tropical Diseases Bulletin* (1952) Saha & Das (1951) though as a rule finding the blood chloride ions in 49 patients examined before treatment to be within normal limits, stated that

"Although there is no appreciable alteration in the plasma chloride concentration the loss of 2.3 litres of body water will be accompanied by the loss of 8 83 gm. of chloride ions (14.5 gm. of NaCl)."

Further observations on this point were made by Chaudhuri and co-authors (1951c) who in the course of an investigation on the body fluid changes in cholera, determined the plasma chloride content of the blood of

the 27 patients studied by them with the aid of the method of Wilson & Ball (1928) They found that the percentage values of plasma chloride were all within normal limits or even slightly increased If however, the total quantity of circulating chloride was estimated from the plasma volume figures and the values were expressed per kilogram of body weight a marked decrease of the chlorides became manifest in all instances particularly in the 10 patients who could not be saved through saline infusions and other means of treatment.

As can be gathered from the tables of Chaudhuri and collaborators, the comparative mean values of total circulating chloride in mg per kg of body weight were 229 8±53 7 in the 17 patients who recovered and 215 8±61 6 in the 10 sufferers who afterwards succumbed In the case of 8 patients who could be examined before and after saline treatment the corresponding figures were 229±32.4 and 362±71

Commenting upon these findings Chaudhuri and co-authors added that,

" as the proportion of chloride concentration is the same in the interstitial fluid as in the plasma, the total loss of chloride from the body is probably much more if we take into account the gross depletion of interstitial fluid that occurs in these cases "

Chakravarti & Chaudhuri (1954) determining with the aid of the usual standard technique the chloride content in the plasma of 17 cholera patients, found—with the exception of one instance of slight hypochloraemia in a sufferer who had developed signs of uraemia on the 15th day of illness—no significant changes in the plasma chloride values They noted in this connexion that similar findings had been made by most previous observers and considered the divergent results obtained by Banerjee (1941a)

" to be due to estimation of whole blood which is much richer in cells in cholera due to dehydration and naturally would give a low figure for chloride as compared to plasma estimations "

As aptly summarized in the *Tropical Diseases Bulletin* (1955) the two authors concluded from their above findings and observations on other electrolytes in the plasma of their patients which will be discussed below, that in cholera the fluid lost from the body was isotonic with the extracellular fluid and that, though the remaining part of the latter was greatly diminished in volume nevertheless its isotonicity was well maintained

(2) Sodium As recorded above (see page 634) Schmidt (1850) examining the blood of 5 cholera patients at varying times after onset of the disease, found the sodium oxide values lowered to 0 111%-0 172% as against a normal standard of 0 190% An identical observation was made *in one* instance by Aron (1910) on a blood specimen from a cholera victim (Na_2O contents 0 129% as against normal values ranging from 0 16% to 0 19%)

Chatterjee & Sarkar (1941) similarly stated that in their 105 patients

"The sodium of the blood serum [determined according to the method of Rourke (1928)] showed greatly reduced figures, especially when the blood concentration is taken into account. After the administration of saline transfusions, as would be expected, there was a normal or even a high sodium content"

As noted earlier (see page 637) the two workers found that in some of their specimens at least the loss of the sodium base exceeded that of the chlorides

In fair agreement with the observations recorded above Saha & Das (1951) found that in 87% of their 49 blood specimens the serum sodium values were below the normal minimum.

Chakravarti & Chaudhuri (1954) with the aid of flame photometry (see Hald, 1947) found plasma sodium concentrations within the normal range in six cholera patients examined before the commencement of treatment. On the 3rd to 5th day after treatment had been started the sodium content in the plasma of the 5 survivors showed a slight reduction, but even then the values were still within normal limits

In a second group of 8 patients whose blood was examined once only after some complication such as uraemia or pulmonary oedema had developed, and who died within the following 24 hours the plasma sodium concentration was usually low However very high plasma sodium values were found in the case of two of these sufferers, to whom rather large quantities of saline had been administered within a short period (12 hours) in an attempt to combat repeated circulatory collapse The two patients succumbed to pulmonary oedema.

(3) Potassium. The early observations made by Schmidt (1850) and Aron (1910) regarding an increased potassium content of the blood in cholera (see page 636) have been confirmed by most subsequent workers paying attention to this problem. Thus Chatterjee & Sarkar (1941) stated that, particularly in the case of severely affected patients, the potassium values, (determined according to the method of Kramer & Tisdall, 1921a) were high, showing in their 105 specimens an average of 25 15 mg per 100 ml of serum. While in some instances the potassium content of the blood became lowered after saline administration "as an after-effect of dilution" no such drop was noted in the case of other patients who had been treated with large amounts of saline and showed a corresponding lowering of the specific gravity of their blood

Saha & Das (1951) recorded that the serum potassium level was above normal in 60 / of their 49 specimens

Estimating the plasma potassium content with the aid of flame photometry Chakravarti & Chaudhuri (1954) found that (*a*) in 4 out of 5 cholera patients who eventually recovered the potassium levels were high on admission, becoming normal or low in the course of infusion treatment and (*b*) in 6 out of the 8 sufferers who afterwards succumbed to uraemia or pulmonary oedema, the plasma potassium values were abnormally high The

two patients in whom these values were within the normal range had been treated within a short period with exceptionally large quantities of saline

Chakravarti & Chaudhuri postulated that the increased potassium content found in the plasma of most of their specimens might have been due to a migration of potassium from the cells provoked by an excess acidity of the plasma and extracellular fluid which the potassium was capable of counteracting. However as justly stated by the reviewer of their paper in the *Tropical Diseases Bulletin* (1955) a more likely assumption was that this outpouring of potassium from the cells was the result of alterations in the permeability of the cell membranes as they were apt to occur in the presence of extracellular dehydration

In view of the identical results recorded by the series of observers quoted above it is rather surprising to find that Ghanem & Mikhail (1949) reached diametrically opposite conclusions in regard to this problem. They stated in this connexion that

"The blood potassium was estimated [with the aid of Kramer & Tisdall s method] in 22 cases on admission. Considering that the normal blood potassium ranges from 16 to 22 mg., and contrary to Chatterjee and Sarkar s statement that the blood potassium increases in cholera, it was found that 18 cases showed hypopotassaemia ranging from 7 1 to 15 5 mg. per cent., three cases showed figures within normal (16 to 17 1) and one case showed a higher figure than normal (22.9) this last patient died "

Ghanem & Mikhail added that

"hypopotassaemia has no relation to the degree of the severity of the clinical condition. Of the six patients who died, the blood potassium was reduced in three, normal in two and in one increased. It can also be seen that saline and glucose infusions were not sufficient to raise the blood potassium to normal."

In the opinion of Ghanem & Mikhail, a potassium loss in the excreta, not compensated for by any intake possibly accounted for the hypopotassaemia they had found in most of their cholera patients

(4) Calcium Loh & Tai (1936) determining the serum calcium content in the blood of 13 cholera patients with the aid of a method devised by Kramer & Tisdall (1921b) found before treatment an average value of 14.3 mg per 100 ml of blood which after saline infusion dropped to 10 1 mg per 100 ml The calcium content of the blood thus appeared to show changes parallel to those of the blood specific gravity

In contrast to these observations, Chatterjee & Sarkar (1941), also using Kramer & Tisdall s method found in their 105 cholera patients before treatment a definite lowering of the serum calcium values, which appeared to be very pronounced if the specific gravity of the blood was taken into account. With clinical improvement, as the specific gravity of the blood became normal the serum calcium values markedly increased, apparently as a rule exceeding the normal figures for Indians (9 5 mg per 100 ml of serum) As the two workers added, it was

" difficult to localize the source from which the increased calcium is mobilized, especially in view of the fact that no calcium is given to the patients either with the saline transfusions or per mouth except perhaps as negligible impurities "

It may be conveniently added that Chatterjee & Sarkar failed to find any constant changes in the *magnesium* content of their serum specimens most of the readings fell within the normal range (3.3 mg per 100 ml of serum for Indians), while in a minority the figures were either higher or lower than the normal average

(5) *Inorganic phosphates* Shorten (1918) determining the inorganic phosphate content in the serum of 11 patients in various stages of cholera according to a method described in his article came to the conclusion that

" The occurrence of phosphatic retention in these cases is definitely established There is some degree of phosphatic retention in every case from the earliest stages, and in untreated cases there is a rise to a maximum and a gradual fall to normal as the patient improves."

As will be discussed below Shorten was led to believe that this retention of phosphates played a most important role in the pathogenesis of post choleraic uraemia.

Liu Wang & Fan (1933b) examining 13 cholera patients found that in the acute stage of the disease there was

" besides the decrease in pH and bicarbonate content a distinct reduction in serum total base and chloride concentration and an elevation of phosphate, and, to a lesser extent, protein."

It was noted that these changes tended to disappear hand in hand with the disappearance of the acute symptoms but that the rate of return to normal of the individual chemical constituents of the blood was apt to vary considerably

Determining the phosphate content of the blood in 13 cholera patients with the aid of a method devised by Benedict & Theis (1924) Loh & Tai (1936) found that, in contrast to the calcium content, an " increase in phosphate has persisted even after transfusion causing an increased demand for base and aggravating acidosis."

Pasricha & Malik (1940) studying the blood of 17 cholera patients, confirmed that in the acute stage of the disease there was an increase in the inorganic phosphate contents As can be gathered from the tabulation of their results, the content of the plasma in inorganic phosphate averaged 7.2 mg per 100 ml as against normal values ranging from 2 mg to 5 mg. The method the two workers used for these determinations was that of Youngberg & Youngberg (1930—see Malik & Pasricha 1940)

Observations on the blood changes in 49 cholera patients by Saha & Das (1951) showed an increased content of the plasma in phosphorus in 75% of the specimens examined in this respect.

Observations on the reaction of the blood and acidosis

It is of considerable historical interest to see that observations on the reaction of the blood in cholera were started as soon as the spread of the disease in Europe facilitated laboratory investigations.

Thus, as referred to above (see page 612) O Shaughnessy (1831 32a) noted an absence or a most marked reduction of the free alkali in the serum of cholera patients

As quoted by Sticker (1912) Buchheister & Noodt (1832) found that the cruor and, to a lesser extent, also the blood serum of severely attacked cholera patients showed an acid reaction and noted such a reaction also in the cerebrospinal fluid of cholera victims According to Sticker Hermann (1832) who made similar findings noted the interesting fact that the acid reaction was still absent in the blood of cholera patients let before the onset of diarrhoea and also observed that the blood of convalescents was no longer acid.

While the validity of the above findings was contested by some of the other early observers, for instance by Wittstock (1832) Garrod (1849) again spoke of a diminished alkalinity of the blood in cholera

An acidity of the blood in the agonal stage of cholera was recorded in 1884 by Straus et al., by Maragliano and by Cantani (see also Cantani 1892) To judge from a remark of Biernacki (1895) a diminished alkalinity of the blood in cholera was noted by Hayem, who presumably referred to this observation in the paper published by him and Winter in 1885

Hoppe-Seyler (1892) mentioned the possibility that the so-called cholera typhoid, which clinically resembled the coma diabeticum was like the latter connected with acid intoxication of the organism Agreement with this opinion was expressed in a note appended to Hoppe-Seyler s article by Quincke who stated that he had actually found a lowered alkalinity in the blood of two cholera patients In one of these a marked increase of the alkali content of the blood was noted after recovery

In the opinion of Biernacki (1895) the lowered blood alkalinity in cholera was mainly due to a decreased sodium content of the blood as established by Schmidt (1850) However an increase in the phosphoric acid content of the blood might also have played a role.

Turning attention to later investigations on the problem at present under review reference has to be made first to a publication by Sellards (1910) who though not making blood examinations, indirectly proved the presence of acidosis in cholera by showing that in this disease a marked tolerance for alkalies existed very large amounts of sodium bicarbonate being required to render the reaction of the urine alkaline As noted by Sellards & Shaklee (1911) an observation on this point had already been made by Quincke (1892) who found that the urine of a cholera patient had remained acid, though in the course of 3 days 30 g of sodium citrate had been administered orally and *per rectum*

Again dealing with the problem of acid intoxication in cholera, Sellards & Shaklee (1911) and Sellards (1914) stated that they had determined the carbon dioxide content in the blood of two cholera patients showing symptoms of uraemia with the aid of the gravimetric method of Kraus (1889) and had found values of 16% and 26% respectively as compared with normal values of 40% to 50% The evidence in regard to the titrable alkalinity of the blood was not conclusive but the results obtained suggested a definite lowering of this (Sellards, 1914) In the opinion of the two workers the acidosis developing in cholera was due not solely to a loss of alkali from the bowel but also to a suppressed excretion of acids by the kidneys.

Rogers & Shorten (1915) determining the alkalinity of the blood in 15 unselected cholera patients according to a method described in their paper found that

"apart altogether from the occurrence of uraemic symptoms, there is a constant reduction of the alkalinity of the blood in cholera which is of a very marked character in all but the very mildest cases, and the degree of which increases steadily with the severity of the disease. A reduced alkalinity of the blood is, therefore, a most essential and important feature of the blood changes in cholera, and one which requires to be combated in the treatment of the disease."

Additional observations indicated an extreme reduction of the blood alkalinity in the case of 5 cholera patients who had developed uraemia.

Once more referring to the subject now under review in his 1921 text book *Bowel Diseases in the Tropics* Rogers summarized the results of observations on 104 cholera patients by stating that

"In only 23 1 per cent. was the alkalinity over N/45 21 of these recovering. In another 23 1 per cent. a moderate degree of reduced alkalinity represented by N/45 but under N/60 was present, and an equal number showed the next degree of reduction from N/60 to under N/80 The percentage of deaths in each of these three classes was 12.5 On the other hand 21 1 per cent. of the cases showed the greatly reduced alkalinity of N/80 to under N/100 with a death-rate of 27.3 per cent., and the remaining 9 6 per cent. of cases gave the extremely low readings of N/100 and less, and their mortality reached the high figure of 60 per cent. one-third of them having died in the collapse stage, and no less than two-thirds, or 67 per cent., of uraemia against 6 7 per cent. of uraemic deaths in the whole series, or ten times as great as the average of the series.

"It is clear from the above data that a greater or less reduction of the alkalinity of the blood occurs in three-fourths of cholera cases, while it reaches an extreme degree in the uraemic ones"

Tsurumi & Toyoda (1922) testing the alkali content of the blood of 49 cholera patients with the aid of a method described in their paper confirmed that there was a relation between the alkali decrease and the severity of the disease The alkali level of the blood was practically normal in the case of 19 patients showing the features of a mild attack and there was no marked decrease in the 11 individuals suffering from cholera in a moderately severe form, only one of whom succumbed. However there was a marked decrease of the alkali content of the blood in the 19 severely affected patients, no less than 13 of whom fell victims to the infection

The modern phase of the investigation of the problem under review may be said to have been initiated by valuable studies on acidosis in cholera made by Liu Wang & Fan (1933a 1933b) Using a method devised by Shock & Hastings (1929) they determined the cell volume pH and total carbon dioxide[1] content of blood samples collected from 28 patients both before treatment and repeatedly afterwards making a total of 222 observations. The serum bicarbonate content of the blood in millimols per litre and the CO_2 tensions in millimetres of mercury were calculated from these values The constant presence of acidosis during the acute stage of the disease was shown by the following findings

> The serum pH on admission averaged 7.28 the bicarbonate content 14 1 and the CO_2 tension 30.7 but the lowest pH observed was 7 07 the lowest bicarbonate content 8.2, and the lowest carbon dioxide tension 16 6 as compared to normal averages of respectively 7 40, 26 6, and 44 9

Following intravenous treatment with saline or alkaline solutions or both, the pH became normal quite rapidly and then the bicarbonate content also returned to normal Irrespective of whether or not they had received alkali solutions, a tendency towards alkalosis soon afterwards became manifest in the 14 recovering patients who could be kept under observation for a comparatively long period However in the four patients who succumbed, the restoration of the serum pH and bicarbonate content to normal through intravenous treatment was but temporary

Studying 48 blood samples, collected from 13 of their patients at consecutive stages of the disease, in a detailed manner (see pages 636 and 642) Liu Wang & Fan (1933b) found that

> " When the values obtained on admission are compared with those on discharge, it may be observed that during the acute stage sufficient base is lost from the body to lower the serum base concentration to the extent of 14 1 milli-equivalents per litre Added to this alkali deficit from loss of base is the increased demand for base from the increase of 2.2 milli-equivalents in phosphate, 2.3 milli-equivalents in protein and 4.5 milli-equivalents in lactate, totalling 9 0 milli-equivalents. The combined alkali deficit from loss of base and from increased demand for it amounts to 23 1 milli-equivalents. This alkali deficit is shared by bicarbonate and chloride. Chloride being a fixed acid, is decreased only 7 0 milli-equivalents, leaving the greater part of the burden to bicarbonate, which is decreased 15 9 milli-equivalents giving as a result marked acidosis."

The following of the subsequent publications referring to the problem of acidosis in cholera deserve attention at the present juncture

Loh & Tai (1936) using the method of Van Slyke & Cullen (1917) found a considerable decrease of the carbon dioxide combining power of the blood in 13 cholera patients examined before treatment had been commenced In contrast to most other initially abnormal properties of the blood in such patients the CO_2 combining power (as well as the non protein

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[1] According to Marcovici (1916) a diminished CO content in the blood of cholera patients had already been demonstrated by Hayem & Winter (1885).

nitrogen content of the blood) remained decreased after saline administration, thus, as Loh & Tai put it suggesting a tendency towards the development of acidosis and uraemia even in the early stage of the disease.

The CO_2 combining power in the blood of 16 cholera patients examined when clinical signs of acidosis and uraemia had become manifest was also found to be decreased, particularly in the samples collected some time after alkaline solutions had been administered

Advocating a modified method of cholera treatment with sodium lactate, Banerjee & Datta (1936) maintained that the development of acidosis in this disease was the result not only of an excessive loss of base in the stools and dehydration of the tissues but also of the production of excess acid by the cholera toxin. However in view of the fact that acidosis is met in a wide variety of morbid conditions including those in which the action of a bacterial toxin is out of the question it is doubtful whether the *V cholerae* endotoxin does play a direct role in the production of this symptom-complex.

As already mentioned (see page 608) Ghosh & Chakraborty (1940) pointed out that the considerable elimination of alkaline base and chlorides in the cholera stools was bound to lead to acidosis and disturbance of the osmotic balance

Banerjee (1941a) referring to the problem of acidosis in his study on hypochloraemia in cholera (see page 637) stated that

"Chlorides play a great part in stabilizing the acid-base balance of the body fluids by helping the interchange of buffer effects between the richly buffered cells and the poorly buffered plasma. In cholera, the acid-base balance is greatly altered (Banerjee, 1936) and so is the quantity of chlorides. This shows that deficiency of the chloride anions in the plasma interferes with the exchange of the bicarbonate anions from cells to plasma.

"A vicious circle thus occurs in cases of cholera as a result of the intimate relations which exist between the loss of chloride and the maintenance of the acid-base balance. If the base and chloride loss remains unrestricted, it leads to but one result and that in the form of depletion of the salt supplies of the body which is invariably followed by loss of water dehydration, retention of nitrogenous waste products and renal failure."

Subsequently the development of an acidosis in severely attacked cholera patients was confirmed by Chatterjee (1946) Safwat & Adham (1948b) and Saha & Das (1951)

The first mentioned worker determining the reserve alkalinity of the whole blood in 55 patients in the collapse stage of cholera with the aid of the method of Levy and colleagues (1915) found this invariably diminished. The decreased alkalinity of the blood was found to persist after the specific gravity and also the phenol content of the blood (estimated according to the method of Theis & Benedict, 1924) had been brought down to normal through infusion treatment.

Continued estimations of the CO_2 content of the plasma in 5 cholera patients as well as occasional determinations made in 12 other sufferers convinced Safwat & Adham (1948b) that there was a marked degree of

acidosis at the onset of the disease, the values becoming gradually normal (50 volumes per 100 ml) when the condition of the patients improved under treatment. Not rarely the CO_2 content of the plasma was initially as low as 22 30 volumes per 100 ml.

Similarly to Chatterjee (1946) Saha & Das (1951) found that there was a depletion of the alkali reserve in the blood of 49 cholera patients examined immediately after admission i e., before treatment had been started The two workers estimated that the average fluid loss of 2.3 litres from the body led to a loss of 28 75 milli-equivalents of bicarbonate ion. Simultaneously with this loss there was a drop in the CO_2 combining power of the blood from 65 to 35 volumes per 100 ml

A further study of the problems presently under review was made by Banerjee et al (1956) with the aim of deciding whether death in cholera was due to acidosis or to an inadequate supply of oxygen to the tissues Therefore the carbon dioxide content of the whole blood and the bicarbonate content of the plasma in 30 cholera patients and in 10 normal individuals were compared with the aid of the methods of Van Slyke & Neill (1924) and Van Slyke, Stillman & Cullen (1919) at the same time the packed cell volume was determined by centrifuging the blood in a Wintrobe tube (see Wintrobe, 1933) Discussing the findings thus made, Banerjee and colleagues stated that

> "The packed cell volume increased significantly in cases of cholera due mainly to loss of plasma. The carbon dioxide contents of the whole blood diminished to a marked degree in cholera cases. Plasma bicarbonate level also decreased significantly in patients suffering from cholera. The diminished alkali reserve of blood indicates a condition of acidemia in cholera The oxygen content of the whole blood and also of the red blood cells in patients suffering from cholera, did not differ from the corresponding values in normal persons. This indicated that cholera patients did not suffer from deficient oxygenation of the tissues and as such death in cholera is not due to anoxemia."

Further reference to the problem of acidosis will be made in subsequent sections of this chapter

Protein content of the blood

To judge from the available information, Garrod (1849) was the first worker who found that in the blood of cholera patients "albumen" was "always in large excess and to this was due in a great measure the increased weight of the serum"

Garrod's contention that the protein content of the blood was increased in the acute stage of cholera has been almost unanimously supported by modern observers The first among these were apparently Liu and colleagues (1933b) who found in the acute stage of the disease in 13 patients an average of 15 8 milli-equivalents of protein per litre of blood as against a mean value of 13.5 milli-equivalents in the 12 patients reaching the stage of convalescence

Using methods described by Malik & Pasricha (1940) for an examination of the blood of 17 cholera patients Pasricha & Malik (1940) established the presence of an appreciable increase in the total plasma proteins due to an increase in the fibrin and globulin fractions Recording their findings in mg per 100 ml of plasma they gave the following relevant figures

	Average	Limits	Normal limits
Protein nitrogen	1538	1078—2139	928—1376
Fibrin nitrogen	94	51— 153	32— 64
Globulin nitrogen	812	495—1348	192— 464
Albumin nitrogen	652	243—1147	544—1072

In contrast to the findings recorded above Ghanem & Mikhail (1949) stated that only 16 out of 23 cholera patients in whom the plasma protein values were estimated according to the method of Phillips et al. (1943) showed a hyperproteinaemia ranging from 7.53 g to 17 4 g. In the other 7 patients the plasma protein values were reduced to values ranging from 3 43 g to 5 65 g, even though in 5 of them a marked or at least a moderate degree of dehydration was present It is noteworthy that in these 7 patients the plasma specific gravity ranging from 1 017 1 027 was invariably not above the normal limits of 1 025-1 028 while it was higher than normal (often markedly so) in the sufferers in whom a hyperproteinaemia was present.

Also using the method of Phillips et al. (1943) for the examination of the blood of 27 collapsed cholera patients, Chaudhuri and co-workers (1951c) found an increase of the total plasma proteins " indicative of the degree of haemoconcentration " Determinations of the total plasma proteins in 8 of these patients before and after treatment gave the following mean values

Before treatment	10 9±1 9 g/
After treatment	6.9±0.2 g/

Thus, as the authors summarized

" From the high values of plasma protein level obtained in these cases, there seems no appreciable loss of plasma proteins from the body "

Further reference will be made to these findings when the merits of plasma administration for the treatment of cholera are assessed

The presence of an increased protein concentration in the plasma of patients in the acute stage of cholera was confirmed by Saha & Das (1951) Lahiri (1951) and Chakravarti & Chaudhuri (1954)

Lahiri (1951) reporting on observations made in the case of 90 collapsed cholera patients, stated that an increase of the total plasma protein was present in many of these sufferers and

" was mainly due to increase of the globulin and fibrinogen fractions, thus altering and often reversing the normal albumin-globulin ratio of the plasma "

Like Liu and co-authors (see page 645) Chakravarti & Chaudhuri (1954) pointed out that the increased amount of protein in the blood of cholera patients

"acts as a weak acid and thereby increases the total acidity of the blood reducing the bicarbonate concentration"

As noted before (see page 641) Chakravarti & Chaudhuri postulated that "potassium from the cell may be called upon to neutralise this rise in the acidity of the plasma"

Blood sugar determinations

Referring to blood sugar determinations in their 13 cholera patients according to the well known method of Folin & Wu (1920) Loh & Tai (1936) stated that

"Another interesting finding was the rapid fall in the content of sugar occurring during the administration of saline, before which the blood was hyperglycemic, more so than what the concentration of the blood could be accounted for and after which it became distinctly hypoglycemic, more so than that due to the dilution with saline. It may be possible that the rise of body temperature which always occurred during transfusion might be responsible for this fall from the increased consumption of available sugar in the blood."

The average blood sugar values in Loh & Tai's series, expressed in mg per 100 ml, were 156 before and 68 after saline treatment. Corresponding cholesterol values, determined in a few instances according to the method of Bloor and co-authors (1922) were 148 mg and 125 mg respectively per 100 ml of blood

Using a method described in the paper of Malik & Pasricha (1940) Pasricha & Malik (1940) found in the blood of 17 patients examined in the acute stage of cholera glucose values ranging from 90 mg to 243 mg per 100 ml of blood (average 162 mg) as against normal values from 60 mg to 120 mg per 100 ml of blood. There was thus an appreciable increase in the glucose concentration

In contrast to these observations Chatterjee & Sarkar (1941) again using Folin & Wu's method, found in 75% of their 105 cholera patients a marked fall in the blood sugar level Commenting upon this result, Chatterjee & Sarkar noted the following

"That there is a fall in the blood sugar in shock like conditions is a well known fact Besides, the great muscular cramps and intestinal movements may have a part in the production of this hypoglycaemia."

Among the 25% of patients in whom no hypoglycaemia was present 4 showed figures above the normal average In the opinion of Chatterjee & Sarkar these comparatively high values could have been due to the blood concentration and possibly also to a non utilization of sugar for unknown reasons

Reference to a low blood sugar content in cholera was also made in the 1943 report of the Indian Research Fund Association.

Again dealing with the problem under review, Ghanem & Mikhail (1949) stated that the blood sugar content, estimated in 21 recently admitted cholera patients by Folin & Wu s method, was lowered in two instances, within normal limits in four and definitely increased in 14 instances, when values ranging from 143 mg% to 280 mg% were found In the case of one patient with an extremely high blood concentration, who died within 24 hours, a blood sugar content of 565 mg per 100 ml of blood was determined. After correction of the dehydration, the increased sugar values returned to normal or low normal (80-134 mg%) in all but one instance in which a sugar content of 65 mg% was noted

Commenting upon these findings, Ghanem & Mikhail stated that

"The degree of hyperglycaemia corresponds more or less to the degree of haemoconcentration as measured clinically and by the specific gravity of plasma also it is known that hyperglycaemia occurs in cases of circulatory failure 12 of these cases with hyperglycaemia showed marked lowering of the blood pressure, and the blood sugar was shown to rise with the deterioration of the clinical condition, including the circulation, in 3 cases under treatment."

The two workers added that in the 6 instances in which the blood sugar content did not rise in relation to the blood concentration, the store of glycogen in the liver and muscles might have been depleted In these instances also the blood sugar content became normal after treatment with not particularly large amounts of saline and glucose solutions

In relation to Ghanem & Mikhail s reference to the glycogen content of the organs in cholera it is important to note that Segale (1912) was unable to demonstrate the presence of this substance in the blood of 6 cholera victims examined soon after death and found only minimal traces in the liver of 2 of these dead bodies. As quoted in the *Tropical Diseases Bulletin* (1912) Segale pointed out

"that many of the conditions known to favour the disappearance of glycogen, such as reduced food-absorption, increased muscular work (cramps), acidity of the tissues and body-fluids, insufficiency of oxygen, fever renal lesions, are present at the same time in an acute case of cholera"

Further blood sugar determinations, made by De and co-authors (1955) in their two groups of patients mentioned above gave the following results

"The blood sugar ranged from 110 to 130 mg. per 100 ml. in 4 cases, from 95 to 105 mg. in 4 cases, and from 55 to 85 mg. in 10 cases of the vibrio-positive group. The mean value was 88 17 mg. per 100 ml. In the vibrio-negative cases of diarrhoea the blood sugar was 120 to 130 mg per 100 ml. in 3 cases, 95 mg. in 1 case, and 60 to 90 mg. in 14 cases, with a mean value of 78.83 mg. per 100 ml. The normal fasting level of blood has been assumed to be 100 mg. per 100 ml."

Commenting upon these findings, the authors stated that

"While some of the cases had normal blood sugar most of them exhibited definite hypoglycaemia. The blood-sugar level was not related to the duration of the disease

during admission The relatively higher blood-sugar level in a minority of our cases may be due to an increase of cortical hormone or to reflex adrenaline discharge in shock However the observation of a low blood sugar in most of the cases is in keeping with the established fact that hypoglycaemia characterizes advanced shock (Sayers 1950). In cholera this finding may perhaps be the result of deficiency of cortical hormone—an excess of which is known to decrease the glucose tolerance."

Observations on urea

A markedly increased urea content of the blood in cholera has been recorded not only by the modern observers enumerated below but also by several of the early workers for instance by O Shaughnessy (1831 32a) Garrod (1849) and Herapath (1849)

Shorten (1918) who as far as could be established was the first among the modern workers to make exhaustive observations on the urea content of the blood in cholera, used for this purpose the urease method of Van Slyke & Cullen (1916) He found that in all his 11 patients

"there was a definite increase in the urea concentration on admission. There was, as a rule, a further increase to a maximum which was reached after a variable time—usually 3 to 6 days. In cases which recover there is a gradual decline to the normal figure"

Shorten emphasized that a high urea concentration was not incompatible with recovery and that, therefore,

"It is not the high urea concentration which is the cause of the symptoms observed in post-choleraic uraemia"

In order to determine the urea content of the blood in their series of 17 cholera patients Pasricha & Malik (1940) used a modification of the colorimetric method of Beattie (1928) described in the paper of Malik & Pasricha (1940) The urea content of the blood of these patients during the acute stage of the disease was found to vary from 28 mg to 125 mg per 100 ml of blood with an average of 62 mg Since the normal values varied from 15 mg to 40 mg per 100 ml of blood, there was thus as a rule an appreciable increase of the urea content of the blood in the limited series of cholera patients examined.

Using a method devised by Mukherjee (1929) for the determination of urea in the blood of their 105 cholera patients Chatterjee & Sarkar (1941) invariably found increased values The two workers insisted that this increase as well as that of the non protein nitrogen (see below) was not due to the concentration of the blood because

"even in those cases in which the specific gravity is restored there still remains the high nitrogenous retention, the figures being considerably high although reduced"

Wilkinson (1943) in a study on cholera in Hong Kong, stated that during the anuric phase of the disease the blood urea content rises rapidly and may reach very high figures Thus one patient, who had been anuric

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for over 50 hours, had at the end of this period a blood urea content of 350 mg per 100 ml of blood. It is interesting that he nevertheless recovered.

Further estimations of the blood urea content were made by Safwat & Adham (1948b) in 63 cholera patients with the following results (showing the maximal rise of blood urea figures)

Number of cases	*Maximal values (mg%)*
25	40-100
18	100-200
12	200-300
8	300-390

In the case of 52 of these patients, the blood of whom could be examined repeatedly the blood urea values became normal in 25% during the first week in 54% during the 2nd week in 15% during the 3rd week, and in 6% later

Reporting also on the results of blood urea clearance tests (made presumably according to the method of Möller and co-authors (1928) described in the textbooks on laboratory technique) Safwat & Adham stated that

"Generally figures obtained run close to those of blood urea, *i.e.*, a minimal value generally accompanied a maximum blood urea value and then urea clearance began to rise to normal value as blood urea decreased. The minimum values obtained were as follows 5%, 18.6%, 22%, 33%, 35%, 39.5% [normal above 70%].

"The time from the onset of the disease to the time when a normal urea clearance value was obtained ranged from 2-20 days. It ran parallel to the time taken by urea to become normal."

It may be conveniently added that the two workers also made estimations of the blood uric acid and creatinine with the following results

"*Blood uric acid* (normal from 0.3-3.5 mgm%) All cases had high values at the beginning. Generally the rise ran parallel to that of urea. The highest value obtained was 11.76 mgm%, the next highest value, however was 8 mgm%.

In all cases when normal values of blood urea were obtained, the uric acid values were at the upper limit of normal range or slightly above normal

"*Blood creatinine* (normal 1-2 mgm%) was estimated in 6 cases only The maximum value obtained was 7.5 mgm%. The next highest value, however was 4.2 mgm%.

"Generally creatinine values closely follow the urea values. It has been noticed in the 6 cases examined that creatinine became normal before urea and uric acid, the time being directly proportional to the amounts retained in the blood."

El Ramli (1948) evidently referring to the group of 52 cholera patients repeatedly examined according to Safwat & Adham (see above) asserted that there was (*a*) no correlation between the haemoconcentration and the blood urea content and (*b*) as shown by 25 observations, also almost invariably no correlation between the chloride and urea contents of the blood in the sense that as the urea values decreased, the chlorides increased. However there was mostly a direct correlation between the blood urea contents and the excretion of the chlorides in the urine, the latter increasing as the blood urea figures decreased.

El Ramli stressed that the blood urea content remained generally high for 1 15 days after cessation of the diarrhoea and maintained therefore with much reason that one

"should not be misled by the hydration of the patient and the absence of diarrhoea and vomiting. One must always think of this high urea concentration and look for it"

Referring to blood urea determinations they had made with the aid of nesslerization (a method going back to Nessler 1856) Ghanem & Mikhail (1949) stated that

"In 17 cases, the blood urea was estimated before and after treatment it was high in 11 the figures ranging from 41 to 179 mg. per cent taking into account the fasting state of the patient. In all the cases the blood urea returned to normal after treatment except in three, where it rose, in only one of these was anuria present"

The two workers maintained in this connexion that an azotaemia, as manifested by a high urea content of the blood, had no relation to the clinical severity of the cholera attacks in question to the blood concentration or to a state of hypochloraemia. It was mainly related to dehydration and circulatory failure but a renal factor of a toxic nature probably also accounted for the production of azotaemia—a postulation which will receive further attention below

Observations on non-protein nitrogen

Recording the results of determinations of the non protein nitrogen content of the blood in 11 cholera patients according to the method of Kjeldahl (1883) Shorten (1918) stated that

"Variations in the percentage of non-protein nitrogen appear on the whole to run parallel to those of urea concentration —A moderate increase on admission, a rise to a maximum in three or four days, and a gradual drop to normal in cases which recover Here again the concentration in the blood bears no relation to the severity of the symptoms, and an increase to a figure many times the normal amount does not preclude convalescence We may therefore exclude the nitrogenous metabolites from the list of possible causes of the symptoms of this form of uraemia [i.e., post-choleraic uraemia]."

In diametrical contrast to this contention of Shorten s, Dhar and colleagues (1930) postulated that, owing to an increase in the non protein nitrogen content in the blood of cholera patients, each instance of this disease was one of potential uraemia.

Using the methods of Folin & Wu (1919) for determining the non protein nitrogen and creatinine contents in 13 cholera patients and that of Folin (1930) for uric acid determinations, Loh & Tai (1936) found that

"The content of non-protein nitrogen, creatinine and uric acid was considerably increased, the increase being more than that due to the concentration of the blood. Even after injection of an adequate amount of saline, their values, though lowered to a certain extent, remained much higher than normal. Two factors may be responsible for this

rise the first, the more important one, is due to anuria in the collapse stage and the second is probably due to the increased protein destruction from dehydration of tissue cells."

Pasricha & Malik (1940) utilizing a method described by Malik & Pasricha (1940) found an appreciable increase of the non protein nitrogen content in the plasma of 17 cholera patients The average values found were 60 mg per 100 ml of plasma (limits 28 145 mg) as against normal values of 18-30 mg.

The observations regarding the subject under review by Banerjee go back to the year 1931 when this worker in a handbook on cholera, drew attention to the marked retention of nitrogenous waste products in the blood of nearly all cholera patients. Though often becoming extreme, the level of these products in the blood became lowered as soon as the urine flow was re-established.

As further quoted by Banerjee (1941) Banerjee & Datta (1935) pointed out that a high non protein nitrogen content or urea retention in the blood of cholera patients did not necessarily indicate a bad prognosis and insisted upon the close relationship existing between haemoconcentration hypochloraemia, and nitrogen retention Which of the two first mentioned factors was of greater importance for the production of nitrogen retention (azotaemia) was a moot question. Banerjee (1939b) inclined to the view that hypochloraemia played a more important role in this respect, because he had noted that introduction of a fairly big quantity of fluid in the form of glucose produced little change in the azotaemia, whereas a small quantity of hypertonic NaCl solution improved both the hypochloraemia and azotaemia

In his 1941 article Banerjee stated that a syndrome called " coma hypochloraemicum " had been described by Porges (1932) in which a diminution of the chloride content of the blood was associated with a rise of the nitrogen content This observation was confirmed by other workers quoted by Banerjee, French authors speaking in this connexion of an *azotémie par manque de sel* (nitrogen retention due to hypochloraemia) Banerjee felt convinced that a hypochloraemia invariably developed in seriously affected cholera patients and, as quoted earlier in this chapter maintained that this salt depletion was apt to lead to a retention of nitrogenous waste products and renal failure

Chatterjee (1941) in an article dealing with the histopathology of the kidney in cholera already referred to in Chapter 6 stated that a retention of nitrogenous products in the blood of patients suffering from the disease was commonly met with, occurring in his experience in 79 % of the cases, while an increase of the blood urea was met with in 72% The retention of the nitrogen substances could not be explained by the mere concentration of the blood because it persisted in patients in whom the specific gravity of the blood had been brought down to normal values through saline

infusions. Chatterjee pointed out that ample observations on a similar retention of nitrogenous substances had been made in shock and allied conditions and that, as defined by Fishberg (1939), this azotaemia, developing even though there was little or no structural alteration of the kidneys, was of a *pre-renal* nature As Chatterjee maintained with much reason the azotaemia in cholera fell into the same category While the retention of nitrogenous waste products was associated with hypochloraemia and decreased renal blood flow in his opinion an action of histamine like substances, as they were produced by *V cholerae* in synthetic media, possibly also played a role in the production of azotaemia.

Supplementing these observations Chatterjee & Sarkar (1941) stated briefly that, as shown by an examination of the blood of 105 cholera patients with the aid of a micro-Kjeldahl method, there was a retention of non protein nitrogen as well as of urea, which remained at a somewhat lowered but still "considerably high" level after the specific gravity of the blood had been decreased through saline administration

Also using a micro-Kjeldahl method, Chatterjee (1946) found in the blood of a series of 55 cholera patients non-protein nitrogen values averaging before treatment 57 8 mg per 100 ml of blood He added that the administration of saline and glucose solutions led to a lowering of these values, giving, however only few details in this respect.

Determining the content of the blood in non protein nitrogen in the case of 61 uraemic cholera patients Tao Woo & Loh (1948) found, in a majority of them, values ranging from 70 mg% to 120 mg% It is noteworthy that there were 6 deaths among the 14 sufferers with blood non-protein nitrogen values ranging from 121 mg% to 270 mg% whereas three fatalities only occurred among the 47 patients with corresponding values below 120 mg% There was no death in the group of 26 patients showing a non protein nitrogen level ranging from 51 mg% to 90 mg% in their blood Thus, as far as these insufficiently numerous observations went, they indicated a relationship between the non protein nitrogen level in the blood of uraemic cholera patients and the outcome of the disease

Commenting upon their observations on the increased content of the blood of cholera patients in urea uric acid and creatinine (see page 652), Safwat & Adham (1948b) expressed agreement with the view "that the azotaemia noted in many cholera cases is of pre renal origin" They pointed out that an analogous postulation had been made in the case of cholera nostras by Froin & Marie (1912)

Lahiri (1951) recording observations on 90 cholera patients, stated that

> "Increase of urea and total non-protein nitrogen of blood was noted in all the severe cases examined and this was evident before there was prolonged anuria."

It is noteworthy that the sodium chloride content in the blood of these patients was invariably found to be decreased

Further reference to the findings recorded above will be made below in connexion with the problem of post-choleraic uraemia

Observations on hyperbilirubinaemia and haemoglobinaemia

As will be further discussed later in this chapter De and co authors (1952) described the case of a cholera patient in whom the presence of a hyperbilirubinaemia and haemoglobinaemia was associated with a haemoglobinuria, and suggested that the appearance of these signs was the result of an absorption of the *V cholerae* endohaemolysin into the circulation, which brought about a destruction of the red blood cells ("intravascular haemolysis") In order to establish the frequency with which such a haemolysis took place De and colleagues (1954b) studied (*a*) 30 cholera patients, in whom the diagnosis could be bacteriologically confirmed and (*b*) 7 patients with clinical signs of the disease, whose stools gave negative results for *V cholerae* It was found that all 30 patients with true cholera showed evidence of hyperbilirubinaemia whereas this was absent in the 7 patients exhibiting only clinical signs of the disease even though they manifested a degree of haemoconcentration paralleling that met with in the bacteriologically confirmed cases Soon after admission, three of the 30 cholera patients showed a haemoglobinaemia with plasma haemoglobin levels of 180 mg, 180 mg, and 138 mg respectively per 100 ml while a fourth examined on the day after admission had a haemoglobin content of 96 mg per 100 ml of plasma. In all these four instances the haemoglobinaemia was found to have disappeared on the day after it was discovered. As De and co-authors added,

"None of the cases of cholera showed any evidence of abnormal susceptibility of the red cells to mechanical trauma, the average fragility [of the erythrocytes] showed no significant difference among the different groups and there was no significant rise in the concentration of sodium chloride at which initial haemolysis took place."

Out of the 4 patients with haemoglobinaemia two also manifested signs of haemoglobinuria, while in a third the first sample of urine (most likely to give a positive result) could not be tested and the fourth died in the stage of shock without having passed urine Only one of the 3 other patients with haemoglobinaemia had an uneventful recovery whereas two like the patient of De and co-authors (1952) eventually succumbed to post choleraic uraemia.

Further reference to these interesting findings will be made in a later section of this chapter

Circulatory Failure

General considerations

In contrast to the views held by early observers it is now generally agreed that the profound circulatory failure which, as will be described in the following chapter forms one of the most characteristic as well as

distressing features of severe cholera attacks is not of a central origin but of a peripheral nature Aptly expressing the now accepted view, Henderson & Seneca (1951) stated that the circulatory failure of cholera

"is of the extracardiac or shock type and is due to extreme depletion of the circulating blood volume It is quite unlike congestive failure for the heart is intact, at least until its function and structure are damaged by anoxia.

It is important to note that the two authors were careful to refer to the *circulating* and not to the total blood volume For though it is certain that the loss of body fluids characteristic of typical severe cholera attacks leads to a reduction of the former it would be erroneous to lose sight of the fact that besides this a change in the distribution of the blood plays a most important role in the production of the circulatory changes manifest in severely affected cholera patients.

As already alluded to in Chapter 6 early attention to the importance of the latter factor was drawn by Simmonds (1892) Like the above-quoted authors, he laid stress upon the fact that, in contrast to other infectious diseases, the myocardium showed no signs of degeneration in victims succumbing in the acute stage of cholera and not very marked alterations in a number of those who succumbed later in the disease, and also maintained that the only striking observation he was able to make in regard to the blood was its unequal distribution, which led to a congestion of the viscera and an emptiness of the vessels of the integument

Similarly Macleod (1910) summarized that in cholera

"The distribution of blood in the body is abnormal the veins and their tributaries are distended with thick dark blood, and the arteries and capillaries empty The solid organs exhibit well-marked venous engorgement.

Similar statements were also made more recently by Chatterjee (1939) who has already been quoted in the sixth chapter, by Banerjee (1941b) and by Ghanem & Mikhail (1949) As Banerjee maintained the circulatory failure resulting in cholera from dehydration, while emptying the arteries led to an engorgement of the veins, particularly of the splanchnic area, and to stagnancy in the capillary system. Ghanem & Mikhail (1949) pointed out with great reason that the capillaries especially those of the intestinal mucosa were capable of retaining several times the normal blood volume Thus in cholera " the effective rather than the total circulatory volume is reduced "

Systemic blood pressure

Ample observations initiated in 1908 convinced Rogers (1921) that in cholera the blood pressure

" is below 70 mm. at the wrist in the majority of the patients on admission to the hospital and commonly below 50 mm. In extreme collapse it is too low to be measured at all at the wrist, such severe cases forming over 40 per cent. of the admissions to Calcutta

hospitals. These observations were mainly made on native patients, whose normal blood-pressure is only from 100 to 120 mm. In European patients equally marked loss of pressure was also met with as a rule.

Commenting on the results of parallel determinations of the blood specific gravity and the blood pressure in 836 cholera patients Rogers added

" that a great decrease in the blood-pressure is of more serious import than a very high specific gravity of the blood Thus, in patients admitted with a blood pressure of over 70 mm. 91 per cent. recovered, and only 9 per cent. were lost, whereas in patients admitted with a blood pressure below 70 mm., 73 4 per cent. recovered and 26.6 per cent. died, and in those with little or no pulse at the wrist, indicated by a blood pressure actually below 50 mm., the mortality was 30 per cent. or over three times that of the first class."

Making daily observations on the blood pressure Rogers also noted

" that if it remained below 100 mm. in adult males or 90 in females for two or three days after the collapse stage was over uraemic symptoms almost invariably developed and proved fatal unless the blood pressure could be raised to over 105 mm."

Occasional instances were met with in which uraemia proved fatal in spite of a blood pressure above 105 mm Hg, but in these patients chronic kidney affections or other pre-existent pathological conditions of the urinary tract (e g. urethral stricture with back pressure effects) were invariably found

The conclusion Rogers drew from these observations will be referred to in a following section of the present chapter

Observations made during the 1947 Egyptian outbreak on 689 cholera patients convinced El-Ramli (1948) that no constant parallelism existed between the blood pressure and the blood specific gravity in their relation to the state of dehydration of the sufferers. It was true that in 71.5% of the patients in whom a high specific gravity of the blood indicated a dehydrated condition the blood pressure could not be measured at all with an ordinary sphygmomanometer or was very low (70 mm Hg or less systolic pressure and no measurable diastolic pressure) However in 28.5% of the patients, even though the blood specific gravity was high, the diastolic pressure was easily measurable and the systolic pressure varied from 80 mm Hg to 130 mm Hg. Thus in this group of patients " the blood pressure could not be taken as a measure of dehydration " On the other hand in some instances in which dehydration, as measured by the blood specific gravity appeared to be slight or even absent the blood pressure was very low or even not measurable at all.

El-Ramli further found that

" The blood pressure is generally corrected either with hydration or even before complete hydration takes place In cases that need repeated hydration the blood pressure fluctuates with the specific gravity of the blood, but in a few of these cases it remains corrected in spite of repeated dehydration. In rare cases in spite of hydration, the blood pressure of patients continues to be low for some time "

Further observations on this point made by Zaki & Ragab (1948) will receive attention in the following section of this chapter

Dealing with the problem of circulatory failure in cholera and its treatment Lahiri (1951 see also Lahiri & Basu 1954) made the following interesting statement

" Circulatory collapse and haemoconcentration due to great loss of body fluid are the immediate results of profuse vomiting and purging in cholera There is initial fall of blood pressure as a result of loss of circulatory fluid in every patient presenting typical signs and symptoms of the disease. In severe cases this fall is profound with imperceptible radial pulse and inaudible sound in the cubital space when recording blood pressure with a sphygmomanometer In many patients immediate replenishment of the depleted fluids and salts produces a quick recovery But in others the circulatory collapse caused by dehydration passes into a condition resembling secondary shock This is commoner in old persons, children and persons otherwise debilitated also in untreated cases and in cases where great loss of fluid has occurred Recovery may of course occur in many patients even when this shock syndrome has supervened but in some cases the collapse persists in spite of all efforts.

In 56 of the patients suffering from such a secondary shock, and who could not be revived through saline transfusion the systolic blood pressure was found to range from 60 to 80 mm Hg, or even less, with an average of 60-70 mm Hg in the majority It is noteworthy that, in agreement with the observations of Rogers, recorded above, in all these patients the specific gravity of the blood was found to have been lowered through the treatment.

Venous pressure and circulation time

Observations on the venous pressure and circulation time in cholera have been made recently by Zaki & Ragab (1948) and by Chakraborty (1954)

As summarized in the *Tropical Diseases Bulletin* (1949), the two Egyptian workers

" found that venous pressure was lowered in all cases and that the degree of lowering was proportionate to the severity of the case Additional factors contributing to the fall in venous pressure were the increased viscosity of the blood, compensating vasoconstriction and loss of muscle tone

Though the arterial pressure was also found to be lowered it showed a less close relation to the degree of clinical severity of the cholera attacks However it was found that the higher the diastolic pressure was the more favourable was the prognosis

The circulation time was found to be but slightly prolonged and its determination was of no prognostic value

According to an exhaustive review in the *Tropical Diseases Bulletin* (1955) Chakraborty studied a series of 60 initially dehydrated cholera patients, 6 of whom eventually succumbed to the disease in spite of saline treatment. On admission the systolic blood pressure in these patients was never above 94 mm Hg and it could not be measured at all in 35 instances

the same was true of the diastolic blood pressure in 46 instances. As noted before (see page 614) determinations of the blood specific gravity in 31 of the patients showed values below 1 065 in 2 instances, from 1 065 to 1 069 in 15 and from 1 070 to 1 072 in 14 cases.

Estimations of the venous pressure were made in 48 of the patients by introducing into a cubital vein an intravenous injection needle connected by a thin rubber tube to a spinal manometer Values definitely above the normal range of 2 10 cm of water i e above 10 cm, were found in 9 instances only, while the average value before saline treatment was 7 5 cm. Infusion treatment led to an increase of the venous pressure averaging 4 cm. After clinical cure values ranging from 7 cm to 10 cm were found in 6 instances, and values above 10 cm in 12 of the convalescents A slight further rise could practically always be noted afterwards.

The circulation time was determined by intravenous injection of 20% dehydrocholine and recording the time after which a bitter taste was first felt in the mouth The average length of the circulation time in the 60 patients was 22.1 seconds as against a mean normal value of 13.5 seconds (limits 9 18 seconds) Values above 18 seconds (maximum over 40 seconds in 2 instances) were found in 46 of the sufferers After saline treatment the circulation time remained unchanged in one instance and was increased in two others In the 57 other patients a drop of an average of 6.3 seconds in the circulation time was noted after treatment. At the time of discharge the circulation time was about normal in 19 instances, while in two it was respectively 24 and 26 seconds.

With four exceptions the venous pressure was within normal limits or but slightly increased when the circulation time was normal.

Renal Failure

Disturbances in urine secretion

That disturbances in urine secretion, manifesting a dysfunction of the kidneys are frequent in cholera and play a most ominous role in the clinical pathology of this disease is generally recognized In fact, as De and co-authors (1954a) recently put it,

> "Even the lay villager of India gives a sigh of relief when the patient passes urine after the day or two of the anuria of the algid stage."

As will be gathered from this statement, a complete cessation of the urine secretion is frequently met with in severely affected cholera patients. The most comprehensive statistical study made in this respect, by Rumpf & Fraenkel (1894) showed that among about 3000 cholera patients in regard to whom pertinent information could be obtained only 698 showed oliguria instead of anuria. That this group of patients with merely dimi

nished urine secretion included many slightly attacked sufferers is proved by the fact that only 33 of them or 4 7% succumbed, whereas there were 590 deaths (57.2%) among 1031 of the patients exhibiting signs of anuria

Depending upon the severity of the cholera attacks i e, upon the rapidity with which the stage of evacuation commences to pass over, or has actually passed over into the collapse stage, the time of onset of anuria varies. As Lebert (1874) stated,

"It has been maintained that in a violent attack the secretion of urine ceases as early as the period of discharges. But it is difficult to prove this statement, and the more attentive patients assure us at times that in the beginning the urine escaped with the discharges from the bowels, so that the quantity could not be noted. Still, it is undeniable that suppression of urine occurs early in some cases."

Generally speaking, however Rogers (1921) was right when stating that

"Suppression of urine ensues as soon as the evacuations have induced a marked fall in the blood pressure it is therefore present for a time, at any rate, in all but the mildest cases of cholera being an important symptom, as it is less frequent and sustained in other forms of diarrhoea."

In relation to the severity of the attacks and the efficacy of the infusion treatment given, the length of time during which the anuria persists may vary considerably Referring in this respect to the experiences of earlier workers as well as to his own observations, Griesinger (1857) stated that in severely affected cholera patients the urine secretion was usually not restored before the second the third or even the beginning of the fourth day from the onset of the attack Similar findings were also recorded by Lebert (1874)

While admitting that generally speaking the restoration of the urine secretion took place hand in hand with the general improvement in the condition of the cholera patients Rumpf & Fraenkel (1894) claimed that the anuria often persisted for days for instance for 12 and 15 days respectively in two patients who eventually recovered, and in a third even for 17 days. Generally speaking, the two workers stated that

"An anuria of 3-7 days is not uncommon either in recovering cases or in those with a fatal issue It is true that the prognosis is more favourable in the instances in which anuria disappears early Nevertheless lack of the urine secretion by no means invariably indicated an unfavourable issue. On the contrary the proportion of those who died though amply voiding urine to the patients succumbing with signs of anuria or oliguria was 4 6." [Trans.]

Rumpf & Fraenkel stressed in this connexion that a lack of urine secretion could not be the cause of the comatose condition developing in cholera patients.

As Sticker (1912) summarized, the anuria, while lasting only 1 2 days in less seriously affected cholera patients, persisted in the severely attacked for 2-4 days.

Modern observers have usually recorded less long a duration of the anuric period in their cholera patients. Thus Loh & Tai (1936) briefly

mentioned that the anuria invariably developing during the collapse stage "may last 30-60 hours" El Ramli (1948) even maintained that in his numerous cholera patients "the excretion of urine became normal on the day following hydration" However Lahiri (1951) studying 90 cholera patients in the collapse stage noted that in those who recovered, urine secretion started after an anuric period lasting from 17 to 58 hours Napier (1946) while stating that a persistence of anuria for 4-5 days was usually followed by death of the patients, referred to the instance of one sufferer who succumbed after he had been anuric for 9 days Napier added that recovery had been seen to take place in patients who had not voided urine for a period of 4 days

As will be further discussed when dealing below with the results of urine examination the urine first voided after the period of anuria is scanty as well as highly abnormal in character However this stage of oliguria is of short duration the quantity of the urine voided per day becoming first normal and then even unusually large so that as some writers put it, a "critical diuresis" takes place during the period of reaction or incipient recovery from cholera.

Even early observers, like Griesinger (1857) and Lebert (1874) felt convinced that, plausible though it might seem at first glance the anuria becoming manifest in cholera patients was not the direct result of dehydration Lebert, though believing that severe cholera attacks led to a degenerative parenchymatous nephritis, admitted that the evolution of this renal affection did not fully account for the anuria commencing as early as the end of the stage of evacuation. He maintained that different factors, like "dryness, diminished arterial pressure distention of the veins anatomical changes in the cortical substance" conduced to the production of the anuria. However like Griesinger before him, he attributed it mainly to the great diminution of the blood pressure in the arterial system.

Liebermeister (1896) dealing with this problem, made the following interesting statement

"A considerable reduction of the urine secretion could be explained satisfactorily by an abundant dehydration It also occurs following the loss of water by other routes, e.g., after excessive perspiration. However whether a complete cessation of the urine secretion, an absolute anuria, is produced through the water loss alone is perhaps doubtful, even though in severe [cholera] attacks the loss of water is more conspicuous and takes place more suddenly than in any other conditions. If however one considers at the same time the severe circulatory failure, one finds a sufficient explanation of the anuria. To ascribe, on the contrary the anuria to an action of the [cholera] toxin on the kidneys is untenable." [Trans.]

Though sharing the belief of early workers like Lebert that cholera led to a nephritic affection of the kidneys, Sticker (1912) declared that during the period of anuria this process was not advanced enough to account for the cessation of urine secretion. He also admitted that even in the most severe forms of glomerulonephritis the urine secretion never ceased so completely

and so suddenly as was the case in cholera Sticker assumed therefore that the anuria characteristic of the latter disease was due to the dehydration of the patients and to an "anaemia in the kidney circulation"

While maintaining "that the failure of the kidneys to secrete in the acute stages [of cholera] is directly due to deficient blood pressure" Rogers (1921) also

"considered it as evident that when the specific gravity of this fluid [i.e. the blood] is much above normal, it will both circulate less freely through the kidneys and also allow of greatly diminished escape of secretion owing to from one-half to two-thirds of the fluid of the serum having been lost. Until the blood has been once more diluted to its normal consistency free secretion of urine is not to be expected."

Several subsequent observers like Banerjee (1941b) Wilkinson (1943) Napier (1946 1951) Lahiri (1951) and Henderson & Seneca (1951) maintained with great reason that the circulatory failure characteristic of the collapse of cholera exerted a most important influence upon the urine secretion by lowering the blood pressure below the minimum necessary for glomerular filtration. This postulation is in accord with the now generally held view authoritatively expressed by Best & Taylor (1955) that

"Anuria occurs when the filtration rate [of the urine in the kidney] is greatly reduced and reabsorption of the small volume of tubular fluid is complete."

At the same time however some of the above-quoted observers as well as other modern workers maintained that the influence exerted by the drop in the blood pressure on the glomerular filtration, important as it was was not the only factor responsible for the production of anuria in the collapse stage of cholera.

Ghosh & Chakraborty (1940) stated in this connexion that the disturbances of the osmotic balance of the blood produced by the loss of alkaline bases and chlorides in the evacuations partly accounted for the suppression of urine observed in the collapse stage of cholera Similarly to this view Wilkinson (1943) drew attention to experimental findings which had shown "that the kidney in salt-deficient animals does not secrete urine freely"

While agreeing with Rogers (1921) that a deficient blood pressure played an important role in the production of the anuria met with in a majority of the cholera patients Chatterjee (1941) postulated that

"Haemoconcentration has also its probable part. The glomerular capillaries are unable to produce a normal volume of filtrate from the abnormal concentrated blood (*Moon, 1938*) Consequently after restoration of blood volume and lowering of the specific gravity of the blood there is again the free secretion *of urine, in the* uncomplicated cases of cholera."

As maintained by Wilkinson (1943) and by Chaudhuri and co-authors (1951c) it was an increased viscosity rather than an increased concentration of the blood, leading—as Wilkinson put it—to a diminution of the minute volume of the kidney which played a role in the production of the anuria.

Tomb (1941 1942) came to the conclusion that a deficient oxygen supply to the kidney accounted for the anuria met with in cholera as well as in other shock conditions

In the opinion of Ghanem & Mikhail (1949) the anuria, met with in only 9 of their 23 cholera patients was partly related to the degree of clinical dehydration. They maintained, however that

" circulatory factors, especially the venous pressure, also play an important role. Analysis of data also suggests a renal element in the production of anuria as well as azotaemia "

In regard to the last of these postulations it is noteworthy that according to Napier (1946 1951) besides haemoconcentration and circulatory failure through loss of blood volume

" possibly some degree of toxic vasomotor paresis with resultant haemostasis lead to failure of the renal circulation, and therefore of renal secretion "

De and colleagues (1954a) asserted, on the other hand that, in addition to the reduction of renal blood flow a cortical *vasospasm* took part in the production of the anuria. This contention deserves greater attention than the postulation of Napier—the more so because as discussed in the sixth chapter it is unlikely that the endotoxin of *V cholerae* exerts a direct action on the kidneys. It is important to consider in this connexion that Best & Taylor thought the ischaemia of the renal cortex, which they suspected of playing a causative role in the production of anuria in shock and allied conditions, to be due to a diversion of the blood through the juxtamedullary glomeruli De & Sengupta (1951) found evidence of such a shunting of the blood from the cortex to the medulla in the kidneys of a cholera victim who had clinically shown signs of profound collapse and anuria.

Results of urine analysis

In view of the limited opportunities for observation and the difficulty of collecting urine samples from patients manifesting the violent and often even uncontrollable purging characteristic of serious cholera attacks, it is not surprising to find that statements regarding the nature of the urine secreted by the sufferers before the onset of anuria are not numerous. To judge from the available evidence (see for instance Griesinger 1857 Liebermeister 1896 Sticker 1912 Napier 1946) prior to its suppression the urine voided by cholera patients is scanty of high specific gravity rich in salts and urates and as a rule contains albumin As added by Sticker casts and epithelia are found in the sediment.

As described by Griesinger (1857) the first urine voided after the period of anuria

" is usually turbid, of a dirty-brownish or deeply yellow colour of low specific gravity (1.007 1 010 according to Lebert, 1856), contains almost always, but not without exception,

varying, often very considerable amounts of albumin [1] is mostly very poor in urea and sodium chloride which latter (owing no doubt to the great salt loss from the intestine during the attack) is often altogether absent The urine forms a sediment consisting of epithelia of the urinary bladder fibrinous [? hyaline] and epithelial casts, leucocytes and not rarely erythrocytes derived from the bladder mucosa, as well as crystals of uric and oxalic acid (Güterbock, 1853) " [Trans.]

Continuing his classical description Griesinger stated that

" The second urine, following most often a few hours after the first, is already abundant and very often already free from albumin If recovery is undisturbed, hand in hand with hydration of the blood as well as with the restoration of the circulation and the metabolism, the urine secretion rapidly increases, so that on the 3rd to 6th day it usually reaches a maximum far surpassing the normally voided amount afterwards the quantity decreases and gradually becomes normal In the abnormally large amounts of urine, quantities of urea far surpassing the normal are voided. On the contrary according to Buhl [1855], the sodium chloride content of the urine only becomes maximal when the urea content decreases according to Güterbock [1853] for the first 8 days after the restoration of the urine secretion the amounts of sodium chloride present cannot be determined by weighing." [Trans.]

Interesting additions to the information supplied by Griesinger were soon made by (a) Begbie (1862) who as quoted by Sticker (1912) " found in the first urine after addition of mineral acids a marked purpuric colour as a pathognomonic sign of past cholera " and (b) Wyss (1868) who definitely proved the presence of large amounts of indican in the urine voided by cholera patients during the reaction stage

Further important observations on the urine of cholera patients were made during the 1892 outbreaks by Hoppe-Seyler (1892) Quincke (1892) Bethe (1892) and Terray Vas & Gara (1893)

Hoppe Seyler (1892) examining the urine of three cholera patients, found a marked indoxyl content, especially in the samples voided immediately after the anuric stage Then the indoxyl secretion soon decreased and disappeared altogether during the stage of " critical diuresis " As shown by parallel examinations of urine samples obtained from patients with gastro-enteritis neither the presence of large amounts of indoxyl nor the increased content of the cholera urines in ethyl sulfuric acid which was also established, was characteristic of cholera alone

Besides proving positive for aceto-acetic acid the urine samples collected from the cholera patients also gave marked reactions for ammonia As Hoppe-Seyler pointed out, the latter result was in accord with the marked secretion of acid in the urines which was connected with the lowered alkalescence present in cholera patients. Reference to a similar observation of Quincke (1892) has already been made in an earlier section of this chapter (see page 643)

As excellently described by Bethe (1892) *the first urine voided by cholera patients after the anuric period*

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[1] According to Griesinger and to Sticker (1912), the presence of albumin in the urine of cholera patients was first demonstrated by Hermann (1832) during the 1830 Moscow outbreak.

" was of the usual straw-yellow colour but invariably very turbid, and on standing formed a thick sediment. The latter consisted microscopically mostly of casts, present in an amount and in a completeness never so far seen by me in any other disease there are extraordinarily long, mostly hyaline casts, but also partly fragments of the straight and convoluted tubules, to which a fatty detritus is affixed. In between are fat-infiltrated renal epithelia and in specially large numbers bladder epithelia, further mucus cells, and leucocytes. Erythrocytes I have found but rarely and then only in small numbers. The amount of casts is of prognostic importance—the larger their number the better is the prognosis The first urine is strongly acid and very rich in albumin. Its amount is small (100-150 ml) but it soon increases, usually reaching on the 3rd or 5th day the normal quantity of 1500-2000 ml. Hand in hand with this increase in quantity goes a decrease of the albumin content, so that usually the urine becomes quantitatively and qualitatively normal at one and the same time." [Trans.]

As further stated by Bethe in all cholera convalescents, regardless of whether they had been seriously or slightly affected the quantity of the urine became further increased to reach amounts far above the normal In ten of his convalescents the daily output of urine increased to 2200-4200 ml. The specific gravity of these urines was maximally 1 012, and in 6 instances 1 007 or less (twice as low as 1 001)

A careful study of the urine of 17 cholera patients (14 of whom had been seriously affected) led Terray Vas & Gara (1893) to the following conclusions

" 1 The so-called first urines are characterized by a small amount, a greenish-brown colour median specific gravity and strongly acid reaction. They contain plenty of albumin and yield a copious sediment, consisting mainly of casts and renal epithelia, but also of leucocytes, more rarely of erythrocytes. The amount of the solid contents, especially of NaCl, Ca, and Mg is markedly decreased. The secretion of urea and phosphoric acid shows only a slight decrease or none at all. Both kinds of sulfuric acid are comparatively increased The amounts of indoxyl- and phenyl-sulfuric acid, of ammonia, and of acetone are also large aceto-acetic acid is also demonstrable

" 2. A diuresis commences in the stage of reaction or even as early as in the stadium typhosum. At the same time the elimination of the products derived from the destruction of the organ tissues commences. Urea, phosphoric acid, as well as ammonia are eliminated in large amounts. The amounts of total sulfuric acid and B-sulfuric acid are maximal in this stage The secretion of NaCl, Ca, and Mg increases incessantly mostly reaching or even exceeding the normal values. Indole, phenol albumin, and acetone are always present in large amounts Aceto-acetic acid is still demonstrable

" 3 The diuresis reaches its maximum in the period of convalescence The urea secretion is still increased likewise phosphoric acid and ammonia are often eliminated in larger amounts. The amount of the total sulfuric acid still shows constantly high values, while the proportion of the two sulfuric acids slowly becomes normal. NaCl, Ca, and Mg have reached or even exceeded the normal values. Albumin as well as the formed elements have disappeared Indole and phenol are present in considerably decreased amounts or are not found any more the same is true of acetone and aceto-acetic acid. In one instance there was a glycosuria lasting for 3 days." [Trans.]

As can be gathered from the text of the article by Terray and co-authors, the maximal daily amounts of urine voided 6-14 days after onset by the patients who had been seriously affected usually varied from 2000 ml to

5000 ml Once the daily output reached the extraordinarily high figure of 7000 ml A maximum of 6000 ml per day was observed in the case of one of the slightly affected patients

Rumpf & Fraenkel (1894) stated that though as a rule the quantity of urine first voided after a cholera attack was small (sometimes only 20-30 ml) occasionally it amounted to 400-500 ml in an exceptional instance a cholera patient even voided after an anuric period of 2 days 3500 ml of urine with a specific gravity of 1 020 Generally speaking, in the experience of these two workers the specific gravity of the first voided urines was not invariably low reaching sometimes values of 1 015-1 020 and de creasing during the following days Albumin was almost invariably present in one patient who eventually recovered the albuminuria persisted for 11 days

Making nitrogen determinations with the aid of Kjeldahl s method Rumpf & Fraenkel found that the N content of the urine was apt to be high in comatose cholera patients who eventually succumbed as well as in sufferers who recovered after having been in a serious condition. In the opinion of the two workers therefore there was no reason to assume that a lack in the excretion of nitrogen was the cause of the coma or of related serious signs manifest in cholera patients

Comparing the results of urine examination in 23 cholera patients who showed signs of moderately severe collapse with oliguria but eventually recovered with those obtained in the case of 6 sufferers who succumbed to uraemia, Nichols & Andrews (1909) recorded the following average findings

	23 recovering patients				6 patients developing uraemia			
Day of illness	*quantity of urine (ml)*	*albumin*	*urea*	*total solids †*	*quantity of urine (ml)*	*albumin*	*urea ***	*total solids †*
1st	18	1.5	0.8	3	17	1 0	0 05	0.5
2nd	188	1 6	2.0	5	10	3 0	0 06	0.4
3rd	725	1 0	12.0	17	50	1 7	0.22	1 1
4th	1023	0.9	17.0	23	60	1 6	0.20	1.3
5th	1337	0 7	20 0	27	153	2.0	0 40	4 0
6th	1764	0.5	24 0	30	125	2.0	1 00	4 0
7th	1810	0.5	30 0	34	160	2.0	2.00	4.0

* Macroscopically estimated with the aid of nitric acid tests

** Estimated with the aid of the ureometer of Doremus

† Estimated with aid of specific gravity determinations

It will be noted that in the recovering patients the quantity of the urine the urea output, and the specific gravity increased while the albumin content showed a decrease In the patients who afterwards succumbed to uraemia, an oliguria continued to be present and was associated with a high albumin content, whereas the urea output and the specific gravity showed only a slight increase

Sellards (1910) performing urine examinations in the course of a study on acidosis in cholera (see page 643) recorded the following observations

(1) Tests for aceto-acetic acid made in the case of 20 cholera patients attacked with different severity and in different stages of the disease gave dubious results, which have

to be considered negative in view of the fact that none of the specimens gave a definite reaction for acetone. (It has to noted, however that these findings are not in accord with the positive findings recorded by some of the above-quoted workers as well as with those of Loh & Tai (1936) mentioned below).

(2) As Sellards stated

"In the examination of 28 cases we have found, almost uniformly an increase in the ammonia coefficient. There were but 6 cases which at any time gave a coefficient of less than 5 per cent, and 3 of these were receiving alkali or acetate in relatively large amounts. In some instances the increase was comparable only to the values obtained in the acid intoxication occurring in diabetes."

(3) As already mentioned (see page 643) Sellards further found that

"Cholera patients showed a definite tolerance to alkalies, a considerable excess of sodium bicarbonate being required to render the urine alkaline as compared with normal individuals. Within certain limits, the administration of alkalies not only failed to render the urine alkaline but its acidity was even increased, as measured by titration."

(4) Also making urea determinations by the hypobromite method in the urine of a number of cholera patients, Sellards found that in the early stages of the disease "a suppression of urea somewhat comparable to the suppression of urine" took place. In the case of patients who were treated with sodium chloride infusions, an increase of the urea output in the urine took place gradually whereas in those treated with alkaline solutions a sudden increase of the urea output was often noted, which was not due to a diminution of the urine flow but was rather accompanied by an increase of the urine volume. Sellards expressed the belief that the initial suppression of urea was not due to a failure of the kidney to excrete urea but was the result of metabolic disturbances created by the acidosis.

As may be conveniently added further examinations of urine samples collected from 50 patients with bacteriologically confirmed cholera and 27 healthy individuals by Chatterjee & Malik (1938) showed in accordance with previous findings, that the reaction of the urine determined with the aid of Hellige s comparator was markedly acid in the acute stage of cholera (pH 4 4-5 4), but became gradually normal as the acute symptoms subsided. On the 7th day after onset of the disease the average pH of the urine samples collected from the cholera patients was 5 9 as compared to a pH of 6 0 in the controls

Attention has to be drawn next to observations by Tsurumi & Toyoda (1922) who stated

"Quantitative tests for chlorids in the urine, were made by Moore s method [*] in ten cases and showed that the quantity of chlorid decreased markedly as the patient s condition reached a very serious stage and increased as he improved, gradually reaching the physiologic state. The quantity of chlorid contained in the urine of a healthy person is about 1 gm. In our patients, the average was only 0.2 gm. on the second day of the disease 0.4 gm. on the fifth day and 0.5 gm. on the ninth day"

According to Takano and co-authors (1926) further observations by Japanese workers showed that the urine passed in small amounts (50-100 ml) by a part of the cholera patients during the acute stage of the disease

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[*] No reference to this method could be found in the available handbooks on laboratory technique or technical dictionaries.

"is strongly acid and contains a large amount of albumin Red blood corpuscles and cylinders may be seen. There is no sugar in the urine at any stage. In many cases, the indican and creatinin reactions are strongly positive"

Interesting results were obtained by Loh & Tai (1936) through examination of urine samples collected during the later stage of the disease from 16 cholera patients, who generally showed a fairly marked degree of acidosis and uraemia and five of whom eventually succumbed

(a) The urine was invariably scanty in amount.

(b) The specific gravity measurable in only 8 of the patients, varied from 1.002 to 1 009 but was almost always below 1 005

(c) Albumin was invariably present and often plentiful

(d) "Acetone bodies" were likewise invariably met with, but tests for them gave mostly weakly positive results.

(e) Granular casts were always found, hyaline casts less regularly

(f) Leucocytes were almost invariably seen in the sediment and were quite often plentiful.

(g) Erythrocytes were found in the sediment in 8 of the patients and were twice plentiful. It is noteworthy that the two patients with numerous red blood corpuscles in their urine succumbed to the disease—presumably to uraemia. Four out of the 6 patients with less plentiful erythrocytes in their sediment recovered.

(h) Phenolsulfonephthalein excretion determined according to the method of Geraghty & Rowntree (1911) was found to be low in all 7 patients tested in this respect.

Further observations on the urine of cholera patients were made during the 1947 Egyptian outbreak by Safwat & Adham (1948b) El Ramli (1948), and Ghanem & Mikhail (1949) The first mentioned two workers, recording findings made in 8 patients, stated that

"1 — At the beginning, all the 8 cases had albuminuria which disappeared with the general improvement of the excretory power of the kidney and drop of blood urea.

"2 — At the beginning all the cases had oliguria which rapidly changed to polyuria that persisted for some time, though the blood urea values were still high.

"3 — Low values of urine specific gravity were obtained, even from small volumes of urine. The specific gravity began to rise only when the blood urea became normal (from 20-40 mgm./%)

"4 — At the beginning hyaline and granular casts were found in 4 of the 8 cases.

"5 — In all 8 cases examined, the chlorides in the urine increased with the general improvement of the case and the drop of blood nitrogenous constituents."

According to El Ramli (1948) if was found that after hydration 80% of the patients who had shown a high blood urea concentration passed normal amounts of urine (about one litre in 24 hours) or even had polyuria. In the remaining 20% oliguria evidently continued to be present In 60% of the patients showing a high urea content in their blood varying amounts of albumin were found in the urine mostly with casts and occasionally with erythrocytes Usually the albumin appeared in the urine of these patients early in the disease but sometimes it appeared only at the end of

to be considered negative in view of the fact that none of the specimens gave a definite reaction for acetone. (It has to noted, however that these findings are not in accord with the positive findings recorded by some of the above-quoted workers as well as with those of Loh & Tai (1936) mentioned below)

(2) As Sellards stated

"In the examination of 28 cases we have found, almost uniformly an increase in the ammonia coefficient. There were but 6 cases which at any time gave a coefficient of less than 5 per cent, and 3 of these were receiving alkali or acetate in relatively large amounts. In some instances the increase was comparable only to the values obtained in the acid intoxication occurring in diabetes."

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[1] No reference to this method could be found in the available handbooks on laboratory techniques or technical dictionaries.

did not pass any red blood cells, while in 2 cases they were detected only on the first day The degree and duration of these urinary abnormalities did not depend upon the duration of anuria (14 to 48 hours) in the stage of shock "

It is of great interest to note that two out of the three cholera patients of this series who, in addition to the above mentioned findings in the urine also showed evidence of haemoglobinuria subsequently succumbed to post-choleraic uraemia While in their case the excretion of blood albumin and casts in the urine was continuous and progressive the urine of the third patient with haemoglobinuria became quickly normal and recovery ensued

Only two instances of anuria lasting for 12 and 36 hours respectively were met with in the controls Albuminuria continuing for 1 5 days seems to have been present in all of them, but some erythrocytes and casts were found only in specimens collected from the two previously anuric patients

Post-choleraic uraemia

It is not surprising to find that the numerous workers who have dealt with the subject of post-choleraic uraemia have reached no agreement as to the etiology of this symptom complex. For as can be gathered from a comparison of the different modern textbooks on pathological physiology and medicine, generally speaking, this question has not been settled. Under these circumstances it is necessary to approach the problem of post-choleraic uraemia in a humble spirit of inquiry rather than with an attitude of certainty

While the early observers were practically unanimous in stating that the post-choleraic uraemia was the result of a retention of urea and other nitrogenous substances normally excreted in the urine, they voluminously debated the relation of this symptom complex to what they called " cholera typhoid " Griesinger (1857) dealing with the latter subject, denied that the patients showing the features of this condition invariably suffered from uraemia. For he said

" there are typhoid conditions in which nothing permits of this assumption and the blood can undergo many other alterations as a consequence of a [cholera] attack (retention of other products of secretion, absorption of components of the exudate, sequelae of the salt loss, etc.) But it is undeniable that an insufficient urine secretion is one of the most important morbid features of this period, and that in a considerable number of cases the presence of an uraemia is sufficiently confirmed on account of the clinical manifestations and the post mortem findings." [Trans]

However Griesinger insisted only those cases fell into the category of uraemia

" in which either the urine secretion did not become restored or soon ceased again, or in which at least but very little urine with a very low specific gravity and a high albumin content was voided " [Trans.]

the first or at the beginning of the second week In 40% of the patients, the urine was normal in amount and free from albumin casts, or erythrocytes, even though the blood urea content was high while the specific gravity chloride content, and urea content were below normal. In most instances the chloride excretion in the urine increased in direct correlation with a decrease of the urea content in the blood.

Determining the chloride content of the urine in 7 recently admitted cholera patients, Ghanem & Mikhail (1949) found values varying from 1.5 g to 10 5 g per litre, but of 3.5 g or less in 5 instances Higher figures were found after infusion treatment. Commenting upon their initial findings, the two workers stated

"Taking into consideration the small amount of urine passed [usually not more than 100 ml], these figures indicate marked salt depletion. In comparing the urine chlorides with the blood chlorides, no correlation could be found suggesting that tissue electrolyte depletion does not reflect itself in the level of blood chlorides. This also shows that blood chloride estimation is fallacious as an indicator of the total loss of these ions in mixed depletion, the urine chlorides are more useful for this purpose, and can be taken as measure of the degree of salt depletion and as a guide to salt therapy"

However regardless of whether or not one accepts these postulations, ample experiences have shown that it is mostly impossible to obtain urine specimens for examination in the early stage of severe cholera attacks. Therefore other criteria have as a rule to be used for the purpose of administering the infusion treatment in an adequate manner

As has been noted earlier (see page 656) De and co-authors (1952) observed haemoglobinuria in a cholera patient who had been admitted in a state of collapse 6 hours after onset of the disease The urine which he first passed 6 hours after hospitalization, showed

"port-wine colour acid reaction, albumin +++ red blood cell ++++ hyaline casts + The supernatant fraction of the freshly voided centrifuged sample showed absorption bands of methaemoglobin and was positive to chemical test for blood."

The urine passed on the following day in a quantity of 528 ml had a deep yellowish colour and a specific gravity of 1 016 red cells +++ granular casts + tests for blood in the supernatant portion of the centrifugate gave a negative result. The urobilinogen content of the urine was high.

Albumin erythrocytes, and casts persisted in the urine of the patient, who succumbed with signs of uraemia on the morning of the 7th day of illness

Examining (*a*) urine samples collected almost daily from 25 patients suffering from bacteriologically confirmed cholera and (*b*) control specimens from 10 patients with non-choleraic diarrhoea (or acute dysentery) with clinical signs of dehydration, De and co-authors (1954a) found that

"after an attack of cholera excretion of albumin and casts is a constant feature and continues for 3 to 17 days. Haematuria lasted for 0 to 17 days—only 3 out of 25 cases

the renal circulation exist to a marked degree It seemed to be a corollary to this contention that it was sometimes possible to save uraemic cholera patients with a blood pressure not exceeding 100 mm Hg through the administration of adrenaline and digitalis

A further noteworthy contribution to the matter was made by Sellards (1910 see also Sellards, 1914) who though believing that cholera uraemia was the result of the appearance of a nephritis pointed out that in this process

"the symptoms of acid intoxication become so intimately related to those of uraemia that differentiation is hardly possible. Indeed it has been suggested by Senator (1902) that uraemia from any cause whatsoever is only an acid intoxication."

Again referring to the relation between uraemia and acidosis Shorten (1918) asserted that

"Post-choleraic uraemia is really a misnomer The condition really is a retention acidosis, as shown by the diminished alkalinity phosphatic retention, and peculiar type of dyspnoea. The concomitant retention of urea and other nitrogenous metabolites does not appear to be of any importance, except insofar as it denotes abeyance of the function of the kidneys."

Though as will be discussed below this postulation deserves serious consideration the authors dealing with the problem of post-choleraic uraemia after Shorten s publication often continued to lay stress upon the pathogenetic role of the retention of nitrogenous products rather than upon that of acidosis. Reference has been made already in this respect to the views propounded by Dhar and co authors (1930 mentioned on page 653 above) A further interesting statement in point by Loh & Tai (1936) was that

"The retention of non-protein nitrogenous substances in the blood, the low sulphonephthalein excretion, the low specific gravity of urine which is always scanty and the presence of albumin, casts, and cellular elements indicate serious damage of the kidneys and profound disturbances of the renal function."

Pointing out that the blood pressure while markedly lowered in the collapse stage of cholera, was usually not significantly low after rehydration the two workers maintained in contrast to Rogers that attempts to raise the blood pressure at this stage with the aid of analeptics were of no value in either preventing or curing uraemia Since both this condition and acidosis were the more likely to become serious the longer the anuria had lasted, a rapid restoration of the urinary flow was one of the main tasks of rational cholera treatment.

Tomb as well as Chatterjee writing in 1941 stressed the importance of a pre renal azotaemia in the causation of post-choleraic uraemia The former author quoted in this respect a statement by Langdon Brown & Evans (1937) according to which, in Tomb s words

"More recently the name non-renal uraemia has been given to the high grade of urea-retention which may develop as a result of non-renal factors. Amongst the most

Lebert (1874) besides mentioning that according to Frerichs (1851) the so-called cholera typhoid was an uraemic condition, also pointed out that

"the later the secretion of urine is established after the third day the graver are the symptoms that may develop from the retention of urea and its transformation into carbonate of ammonia some of these patients die of exhaustion and others with the typhoid manifestations of uraemia."

Another point emphasized by Lebert was that, as far as his experiences went, the kidney affection of cholera almost never became chronic—an observation confirmed by all subsequent workers

Lebert s claim as to the frequency of uraemia in those patients who had been anuric for a long period was supported by observations of Rogers (1909b) For as the latter worker stated an uraemia was particularly often met with (*a*) in cholera patients admitted more than 48 hours after onset of the disease, the early treatment of whom had been consequently neglected and (*b*) in sufferers who had been attacked with particular severity so that—in spite of early commenced treatment—it was extremely difficult to restore the urine secretion Thus as Rogers added in 1921

"In both cases there has been prolonged suppression of urine and stasis of the renal circulation, making the restoration of the functions of the kidneys a difficult matter"[1]

It was in accord with these observations that, as Rogers (1909b) stated, signs of uraemia appeared more commonly in those cholera patients whose systolic blood pressure could not be raised above 100 mm Hg Further examining the kidneys of patients who had died in the uraemic stage of cholera, he was

struck by the amount of effused blood in and around the convoluted and straight tubules and the tense state of the capsule enclosing the extremely congested organ all suggesting an actual mechanical difficulty in the re-establishment of an efficient circulation through the organ."

"In order to test if this was the case or not," Rogers continued, "I tried perfusion of normal saline solution through the renal artery from different heights, so as to measure the actual pressure required to obtain a fairly full outflow from the renal veins."

He established in this manner that, while a pressure equal to 20-30 mm Hg sufficed to perfuse the kidneys of subjects who had died of other causes, a pressure of 90-100 mm Hg was necessary to obtain the same result with the kidneys of victims to post-choleraic uraemia. In one of the latter experiments, subsequent splitting of the renal capsule reduced the pressure required for perfusion to 20 mm Hg. Since, moreover a pressure of 30 mm Hg sufficed to perfuse the kidney of a cholera victim who had died of a complication after the urinary flow had become re-established, Rogers felt convinced that only in post-choleraic uraemia did an obstruction of

[1] It is interesting to note that views similar to those of Rogers have been expressed quite recently by Chakravarti & Mondal (1956) Their conclusion was that in cholera two types of renal reactions might be encountered. Firstly (in the majority of cases) there might be initial renal failure due to quickly reversible functional disturbance following extreme fall of blood pressure and possibly due to compensatory renal ischaemia In the second group, during the initial shock stage, the ischaemic process may be acting too long to precipitate variable organic damage of the kidney

Recording observations on 689 cholera patients, El Ramli (1948) maintained that only a minority of the sufferers with a high urea concentra tion in the blood developed " the well known classical signs and symptoms of uraemia " and that " their prognosis was worse than that of the others " Some others did not show signs except a slight weakness, while the majority of the patients with a high blood urea level

" were found to be easily fatigued with slow cerebration. The tongue may be dry or moist, the appetite is bad slight diarrhoea and a slight hiccough may be present but vomiting is rare. The urine in most of the cases is normal in amount and in a good proportion, free from albumin."

Such patients rapidly improved as the urea content in their blood decreased and apparently they became well before the blood urea level became normal

Discussing the problem of post-choleraic uraemia in an interesting article to which repeated reference has already been made in this chapter Chakravarti & Chaudhuri (1954) laid stress upon the fact that the plasma potassium level as well as the urea concentration of the blood were high in a group of 8 patients who succumbed to uraemia, or pulmonary oedema or both, thus supporting the assumption that the much raised potassium concentration in the plasma was a contributory cause of death In two further patients, who could be cured even though they had developed uraemia, the initially very high potassium and urea levels came down to normal with recovery the plasma potassium levels even temporarily reaching subnormal values in one of these two convalescents

On account of the findings recorded above (see page 671) De and his colleagues (1954a, 1954b) came to the conclusion that the initial haemoglobinaemia and haemoglobinuria observed in some of their cholera patients might have played an important role in the subsequent development of uraemia.

For an evaluation of this postulation reference has to be made to a statement by Rogers (1921) according to which post-choleraic uraemia was particularly frequent in what he called the haemorrhagic form of the disease Rogers evidently used this term to designate instances in which haemorrhagic stools were found during life and extensive haemorrhages were seen on the intestinal mucosa, especially that of the caecum at autopsy However Rogers also drew attention to extensive haemorrhages in the kidneys apparently met with only in the cholera victims who had succumbed to uraemia.

It has also been noted (see page 669) that Loh & Tai described two in stances in which apparently fatal post-choleraic uraemia was associated with the presence of numerous erythrocytes in the urine.

Still, while these earlier findings lend indirect support to the postulation of De and co-authors, the observations made by the latter workers are not

important of these factors are *vomiting* and *diarrhoea* both of which cause urea-retention through loss of body fluids and the accompanying loss of chlorides."

Chatterjee (1941), while admitting the paramount importance of the pre renal azotaemia, considered it possible that the histological changes (swelling of the basement membrane of the glomeruli and the tubules accompanied by non inflammatory dilatation of the capillaries of the glomerular tufts and the medulla) detected by him in the kidneys of uraemic cholera victims because they impeded filtration might to some extent be co-responsible for the high level of nitrogenous products in the blood

Commenting upon the case of a patient who showed clinical signs of post-choleraic uraemia associated with a high level of non protein nitrogenous products in the blood, but with a urine specific gravity of only 1 010 Chatterjee maintained that possibly this low specific gravity of the urine could be explained

" by the functional disturbances of the tubules and their inability to concentrate urine and absorb water owing to the great thickening of the basement membrane as well as the engorgement of the capillaries "

As further stated by Chatterjee, there was no reason to assume that pre-existing kidney diseases frequently accounted for the appearance of post choleraic uraemia such chronic kidney alterations were met with only in two of the 13 uraemic cholera victims examined by him Moreover 78% of these victims were less than 35 years old. Similar observations were afterwards recorded by Wilkinson (1943) who found a uraemic condition ascribed by him to acidosis, particularly frequent among " stocky broad-shouldered young men " It is noteworthy however that in contrast to these experiences Henderson & Seneca (1951) maintained the incidence of post-choleraic uraemia to be higher than usual in patients with damaged or overburdened kidneys, e.g., in pregnant women Rogers (1952) besides stating that in his experience fatal post-choleraic uraemia was most frequently met with " in feeble Indian subjects of fifty years of age and upwards " also asserted that

" A very slight degree of chronic interstitial nephritis, requiring an abnormally high blood pressure to produce sufficient renal secretion in a healthy subject, is another not rare cause of fatal post-choleraic uraemia. Such fibrosis of the organ is met with much more frequently in fatal cases of cholera than in the general run of autopsies "

Safwat & Adham (1948b) though entitling their valuable article *Uraemia in cholera* dealt not so much with this condition as with azotaemia. Since, examining the kidneys of a few uraemic cholera victims they found marked histological changes, they did not wish to exclude the possibility that the latter might have played a causative role in the production of azotaemia. Nevertheless, they admitted that " the azotaemia noticed in many cholera cases is of pre renal origin "

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numerous enough to permit of a decision as to whether post-choleraic uraemia stood to the haemoglobinaemia and haemoglobinuria in a causal or merely in a *post hoc* relationship

Turning from the rather discrepant views of the cholera workers quoted above to the modern literature on uraemia in general one finds a similar diversity of opinion Some of the recent writers have adopted the thesis propounded more than fifty years ago by Senator (1902) that acidosis played a preponderant role in the production of the uraemic symptom complex. Thus Best & Taylor (1955) stated that the symptoms of uraemia

> are now believed to be due rather to a general disturbance in water and electrolyte metabolism resulting in serious abnormalities in the chemical composition of the body fluids. Acidosis and an imbalance of certain electrolytes, especially of sodium, chlorine, calcium, phosphorus and bicarbonate, leading to a disorder in osmotic relationships and in the distribution of intracellular and extracellular water are looked upon as the causative factors."

Most of the modern writers, however still ascribe uraemia to a retention of waste products or to the presence of an azotaemia. While it is now often held that the nitrogenous products normally excreted in the urine do not play a role in this respect, it does not seem that any other harmful substances have been definitely incriminated.

Some observers maintain with much reason that the symptom complex of uraemia is due to an interaction of various causative factors rather than to any single cause Mason & Harrison (1954) aptly insisted in this respect that

> "In the production of the manifestations of uraemia, two factors are particularly concerned. One of these is *excess* of certain substances, of which the nitrogen-containing compounds of the urine, the phenolic bodies, phosphates, potassium, water and the acidic ions are perhaps the most important. The other is *deficiency* of certain substances. Such deficiencies arise secondarily as the result of either retention of chemical antagonists, or of loss by the kidneys or by other routes. Thus retention of non-volatile acids, defective ammonia formation and failure to conserve sodium all tend to cause deficiency of alkali reserve indicated by a decline in serum bicarbonate or total CO_2 "

As far as the present writer can judge, this general hypothesis adequately accounts for the pathogenesis of post-choleraic uraemia. When dealing with the latter great attention ought to be paid as well to the additional dictum of Mason & Harrison that

> "Since it is easier to remedy a deficit than to overcome an excess, search for deficiencies of essential components of the body fluids is of special practical importance in patients suffering from uraemia."

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Chapter 9

SYMPTOMATOLOGY, DIAGNOSIS, PROGNOSIS AND TREATMENT

SYMPTOMATOLOGY

Incubation Period

Dealing with the length of the incubation period in cholera, Griesinger (1857) pointed to the marked discrepancy of the findings recorded in this respect while some observers spoke of an average length of the incubation period of one to two days, or 50-60 hours, with a maximum of six days, others claimed that longer intervals, even up to three or four weeks, could separate the time of infection and that of outbreak of the disease. While considering the existence of such long incubation periods with scepticism Griesinger pointed to

"numerous instances showing that the incubation period can be very short, cholera breaking out 12 24 hours after the first possibility of infection. There are still more numerous, indeed very many instances where the length of the incubation period seems to have been 2-4 days " [Trans.]

A further important statement by Liebermeister (1896) was that

"There are cases in which the disease breaks out within 24 hours following an opportunity of infection On the other hand, several days may elapse from the time of infection until the appearance of marked [*ausgebildeten*] manifestations, in rare instances even 8 or 14 days or more in the latter cases, a moderate diarrhoea sometimes already existed for a longer time, but was neglected, until suddenly perhaps on account of a gross error of diet, the severe signs appeared. [Trans.]

Liebermeister felt convinced that usually the incubation period of cholera, averaging two to three days, was much shorter than that of most other infectious diseases Indeed, it was, in fact, still shorter than was often assumed, if as was justified, account was taken not of the onset of signs of a severe attack, but of the appearance of a prodromal diarrhoea. He also drew attention to observations made by Pettenkofer (1856) during the 1854 cholera outbreaks in Bavaria, which had shown that

(*a*) Five persons reaching a cholera-free place from an infected locality fell ill two and a half to five days after arrival and

(*b*) In 18 instances an average of seven to eight days elapsed before contacts of persons who had recently arrived from a cholera focus in a hitherto uninfected locality manifested the disease

Commenting upon the latter observations Liebermeister again insisted that in most of these instances the actual length of the incubation period was shorter than that recorded because there was no reason to assume that infection invariably took place at the first opportunity

In the opinion of Macleod (1910) the duration of the incubation period in cholera

> varies from a few hours to a few days, probably not exceeding ten. Three to six days appear inferentially to be the usual length of this stage "

Sticker (1912) discussing this problem drew attention to a report rendered in 1832 by the British Central Board of Health wherein it was asserted on account of numerous observations made at home, in 18 ships which had arrived from the Baltic Sea and in India, that cholera broke out most often on the first day after infection then frequently within the second to fifth days, more rarely on the sixth day and quite exceptionally still later

Like the above-quoted authors, Sticker was sceptical regarding the existence of unusually long periods of incubation pointing out that in such instances infection might have been contracted from some intermediary source, e g., from cholera carriers

Further most interesting observations on the length of the incubation period in cholera were recorded by Babes (1914) who stated in this connexion that

(*a*) Numerous experiments had shown that cholera infected animals fell ill as early as the day following the infection similarly to the maximal growth shown by *V cholerae* after an incubation of 24 hours

(*b*) Human beings who voluntarily or involuntarily contracted the infection through ingestion of material from cholera cultures invariably showed signs of the disease on the first or second day following the entry of the organisms into the gastro-intestinal tract and

(*c*) As shown by ample experiences in Romania under natural conditions as well the incubation period in human cholera hardly reached a length of two days

Babes added that

> " In numerous cases we examined isolated persons every 3-5 days for the presence of *V cholerae* and never found the organisms earlier than [about] 24 hours before onset of the disease. If vibrios were found, either one had to do with carriers, or the positive result immediately preceded the onset of the disease (12, 18 26 hours before onset) " [Trans.]

As summarized by Takano and co-authors (1926) Murayama (1917) found incubation periods ranging from 18 hours to three days in 207

seriously affected cholera patients Kamimura & Tsuda (1921) recorded in four fifths of their cholera patients an incubation period of less than five days, and in a majority one of three days. According to their observations the onset of the disease took place more rapidly in children under 10 years and in females than in adult males

The views held by modern observers regarding the length of the incubation period in cholera may be exemplified by the following statements

Author	*Length of incubation period*
Strong (1944)	Usually 1 5 days, more commonly not over 3 days.
Napier (1946)	Not longer than 5 days, usually less than 3 days. Sometimes the first symptoms appear within 24 hours after infection.
Henderson & Seneca (1951)	1 5 days, usually 3 days. " A very brief incubation period, *perhaps only a few hours, is occasionally found during* an epidemic, but its significance is difficult to determine."
Shattuck (1951)	Varying from a few hours to 5-6 days, but usually about 3 days.
Manson-Bahr (1954)	" Although cholera may declare itself within a few hours of exposure to infection, it may also do so at any time up to ten days. Three to six days may be set down as the usual incubation period."

It is significant that most of these authors considered a period of up to three days as the usual length of incubation in cholera and that according to most of them the interval between infection and onset of the disease was never longer than five or at the most, six days. Only Manson Bahr influenced presumably by the statement of Macleod (1910) quoted above and possibly also by observations recorded during the 1947 Egyptian outbreak,[1] spoke of a period of up to 10 days, whereas Henderson & Seneca (1951) though in general agreement with the other writers quoted above, maintained that if

" the ingestion of vibrios took place long before the onset, which was precipitated by intercurrent disease (e.g. dysentery), then the incubation period must have been the duration of the carrier state, or a matter of weeks."

Since, however one must assume that, ordinarily at least, carriers of *V cholerae* do become immune to this organism, the occurrence of an activation of the infection postulated by Henderson & Seneca appears to be an altogether unlikely contingency It seems legitimate therefore, for all practical purposes to reckon with an incubation period not exceeding five or at most, six days in the case of cholera. The former figure (5 days) has been adopted for the purposes of the International Sanitary Regulations promulgated by the World Health Organization in 1951

[1] Kamal (1951) claimed that among 123 quarantined contacts of cholera patients 16 fell ill with the disease on the seventh to tenth day of isolation

Clinical Types

Defining the usual characteristics of the disease, Griesinger (1857) stated with admirable conciseness and exactness that

"The process of cholera appears in the form of an attack with a quick and feverless course, in which colourless evacuations, vomiting, cramps, collapse, cessation of urine secretion, disappearance of the pulse, algor and cyanosis are the principal phenomena." [Trans.]

However Griesinger continued

"these symptoms are peculiar to marked cases and as in the other infectious diseases, e.g., the various forms of typhus, there are many differences of gradation in the development of symptoms, inasmuch as the specific cause produces either a violent or a very slight disease. It has been generally agreed, therefore, to distinguish between various actually occurring forms [*in der Natur begründeten*], which differ first of all in degree" [Trans.]

These separate forms were (*a*) choleraic diarrhoea (*b*) "higher degrees of intoxication" designated as cholerine and (*c*) the typical syndrome of cholera. Later in his text Griesinger also referred to instances of a *foudroyant* nature, without, however, separately classifying this rapidly fatal type as cholera sicca or less commonly but more felicitously as cholera siderans as many other authors did

Accepting Griesinger's classification, Lebert (1874) aptly wrote

"We include under the term of Asiatic cholera the diarrhoeas which occur during the prevalence of an epidemic, cholerine and the well-defined grave forms of the disease. That all three are expressions of the disease itself and not simply degrees of it, is proven by the facts that the simple diarrhoea often terminates itself without leading to cholera that the grave forms of cholera not infrequently begin precipitately without previous diarrhoea and, finally that cholerine occurs mostly without previous diarrhoea, frequently does not turn into cholera, and may present, in the most positive manner all the signs of the graver forms—only a few of these signs, of course, being present in one and the same case."

Sticker (1912) one of the authors putting cholera sicca, siderans or "apoplectica" in a separate group aptly designated cholerine as "cholera minor" (*kleiner Choleraanfall*) so as to differentiate it from the "grave" or "major" form of the disease Were it not for the undesirability of using unfamiliar terms, the name of cholera minor would be preferable to that of cholerine because the latter is sometimes incorrectly used to designate gastro-intestinal affections not caused by the *V cholerae* or—*horribile dictu*—even the prodromal diarrhoea held by some observers to precede typically severe cholera attacks The term "ambulatory" cholera used by some modern writers to designate attacks of choleraic diarrhoea has also found no general acceptance Under these circumstances it seemed best to adhere for the purpose of the present disquisition to the classification adopted by the above-quoted early authorities It has to be kept in mind,

however that no sharp lines of distinction can be drawn to separate choleraic diarrhoea from cholerine or the latter from the grave form of the disease More than that, nor can a sharp distinction be made between the slightest forms of clinically manifest cholera and what some authors—first, possibly Rumpel (1894)—called cholera infections without clinical manifestations (*Commainfectionen ohne klinische Folgen*) to designate instances in which *V cholerae* had been isolated from the stools of apparently quite healthy persons, i.e., from carriers of the infection.

Rumpel claimed in this connexion that the absence of clinical signs in the nine carriers detected by him, as well as that of severe cholera manifestations in the 60 patients with choleraic diarrhoea or cholerine observed by him was the result of a " personal " immunity of the individuals in question. Though they had been hospitalized, they received no treatment and there was also no reason to assume that an onset of grave cholera had been prevented through the adequate diet they had been given because most of the 82 patients suffering from the latter form of the disease had not committed any manifest errors in their diet before they fell ill

It is important to add that, before the period of 1892 94 both Gruber (1887) and Lustig (1887) had confirmed the validity of the clinical observations of the earlier workers through demonstration of sometimes enormous numbers of cholera vibrios in the stools of patients manifesting merely signs of slight diarrhoea

Choleraic Diarrhoea

Comparing the descriptions of choleraic diarrhoea by different authors one is struck by the discrepancy of the data, proving that at least three different subtypes of this slightest form of cholera may be distinguished—namely (1) a quite mild affection manifested only by diarrhoeic evacuations (2) a transitory form in which other signs as well are present but not conspicuous and (3) a comparatively more serious form, in which the latter signs though still overshadowed by the diarrhoea, are more prominent. It is, however important to note that even the patients more seriously affected in this way do not vomit, and that, though more violent choleraic diarrhoea may lead to a diminution of the urine secretion and occasionally also to albuminuria, it never produces the anuria characteristic of grave cholera.

The number of evacuations in choleraic diarrhoea and the duration of this affection may vary considerably Reporting upon 25 observations, Rumpel (1894) stated in this respect

* The diarrhoea was of varying intensity from a single evacuation throughout the illness to 20 dejections on one day The aspect of the dejections was most often that of an ordinary diarrhoeic stool [*Dünndarmstuhl*], but one also saw typical rice-water stools. The duration of illness varied from one to 24 days." [Trans.]

The appearance of rice water like stools in choleraic diarrhoea appears to be exceptional However it deserves attention that as stressed by Guttmann (1892) the more or less fluid and bile-coloured dejecta ordinarily met with in this type are rich in mucous flocculi, in which the causative organisms are particularly abundant. Guttmann admitted, however that such mucoid stools were also observed in cholera nostras and in dysentery

A classical description of the comparatively most serious subtype of choleraic diarrhoea as well as of its slighter forms was given by Griesinger (1857) thus

"In this affection there is a daily evacuation of 2-8 thin, faeculent-mucous, bile coloured stools, usually first during the night or in the early morning the evacuations are accompanied by flatulence and particularly by borborygmus in the lower abdomen, but hardly or not at all, by abdominal pains. Often the patients feel quite well and have an appetite in very many cases, however the diarrhoea is associated with a white coating of the tongue, a sticky taste in the mouth, thirst, a sense of pressure in the stomach, nausea, often marked malaise, lassitude, headache, ringing in the ears, diminution of the urine secretion, and twinges in the calves of the legs there may be also a tendency to coldness of the extremities or to abundant perspiration, or slight fever may be present." [Trans.]

While stating that gradual recovery was by far the most frequent outcome of choleraic diarrhoea, Griesinger maintained that

"In old, markedly weakened persons or in small children, or under extremely unfavourable environmental conditions, e.g., among soldiers in the field, there may be a fatal termination, without transition into the grave form of cholera, with signs of exhaustion the patient becomes weaker from day to day he looks more collapsed and grey and children and quite old people in particular die within a period of up to 8 days without showing signs of typical cholera, except perhaps traces of muscular cramps." [Trans.]

With all due respect to the clinical acumen of Griesinger one might claim that these patients suffered from atypical major cholera rather than from choleraic diarrhoea, which by general consent is considered a benign form of the disease, terminating in recovery even without treatment.

While maintaining with prophetic wisdom that choleraic diarrhoea was etiologically different from all other forms of diarrhoea, Griesinger admitted the impossibility of definitely differentiating between the former and the latter on clinical grounds He pointed out, however that choleraic diarrhoea was often more persistent and, since it impaired the general condition of the patients more was more apt to lead to a retarded recovery Borborygmus and the slight disturbances noted above on the part of the nervous system were also more characteristic of choleraic than of ordinary diarrhoeas.

To determine the comparative frequency of choleraic diarrhoea is well nigh impossible. Dealing in this connexion with the early manifestations of cholera in Europe Sticker (1912) referred to a high incidence of diarrhoea or of other minor gastro-intestinal disturbances preceding and accompanying the outbreaks He admitted, however that

"these manifold morbid conditions were not rarely *morbi facilili* produced partly through sudden changes of the usual diet, particularly the daily consumption of red wine and a strictly animal diet, and partly through constant fear care and other mental emotions, but most often no definite predisposition could be determined. The spread of this predisposition corresponded throughout to the increase and decrease of the epidemic, so that it was much less manifest in the last weeks of decline of the disease." [Trans.]

It is most noteworthy that the case incidence of diarrhoea was sometimes quite out of proportion to that of typical cholera. Thus, according to Kopp (1837) at the time of the 1836-37 epidemic in Munich, which claimed 915 victims among a total of 1974 patients with cholera gravis, more than 12 000 sufferers from diarrhoea were medically treated According to the 1850 report of the British General Board of Health, also quoted by Sticker there were, in 1848, 600-4000 diarrhoea patients per 100 cholera patients. In the following year 130 000 diarrhoea patients were treated in 15 major English towns as against 250 patients with cholera gravis.

Writing after the discovery of *V cholerae*, Cantani (1892) made the following statement

"Whoever has taken the trouble to search during a widespread cholera outbreak in the stools of persons suffering apparently from simple diet-caused diarrhoea for cholera bacilli, will have found ample evidence that during such an epidemic there occur very numerous cases of apparently simple, spontaneously terminating or easily curable diarrhoeas due to the comma bacilli " [Trans.]

Cantani added however that these patients, if neglected by their physicians, often developed signs of grave cholera. It is not possible, therefore, to deduce from his statement the frequency of uncomplicated choleraic diarrhoea Reliable information regarding the latter was furnished by Rumpel (1894) who had an opportunity of observing 317 persons kept in quarantine as cholera suspects 151 of these individuals were found to be infected with *V cholerae* thus

	Number	Percentage
Healthy carriers	9	6.0
Choleraic diarrhoea	25	16.5
Cholerine	35	23 1
Cholera gravis	82	54.3
Total	151	99.9

According to these figures the case-incidence of choleraic diarrhoea was not as high as the general statements of some other authorities would lead one to assume. Nevertheless, even according to these statistics, the occurrence of this form of cholera, which on account of its banal manifestations is apt to escape the attention of the medical staff under ordinary circumstances, was quite considerable enough to deserve most serious attention.

Cholerine

In order to deal adequately with cholerine it seems best to quote the excellent description of this form of cholera by Lebert (1874) who stated

"that the cholerine which appears during the prevalence of a cholera epidemic is only a mild form of cholera, a statement which finds additional support in the fact that some of the grave symptoms of cholera are often associated with cholerine. According to the histories of cases which I have collected at different times, a diarrhoea lasting from one to three days preceded the attack of cholerine in one-eighth of all the cases. Cholerine is, on the whole, a condensed picture of a true cholera attack in its mildest form. Malaise, headache, weariness of the limbs, diminution of appetite precede it for one or two days, or at least twelve hours. The attack of cholerine occurs generally in the night. Patients sleep with discomfort are restless, and are then suddenly awakened by the necessity for a stool. Copious yellowish-brown, almost watery discharges follow each other at short intervals, to the number of three, four eight, or even twelve or more, until in many cases where the discharges are very numerous they become at last colorless and like rice water During the very first operations patients complain of fulness and tension in the praecordial region, with nausea soon afterwards vomiting occurs, at first of the remains of the food, then of a yellowish-green, very fluid, bitter or sour substance. It is thrown up not unfrequently by an easy regurgitation, and its great quantity at once reminds one of the vomiting of cholera it may indeed, at last become colorless and whey-like, and show a deposit very much like bruised grains of rice. The vomiting is repeated quickly four or five times, then becomes more infrequent, less in quantity and ceases altogether in a few hours. The patients, who have in the meantime become very much reduced, now either recover rapidly or the inclination to diarrhoea continues for some days, with lack of appetite, meteorism, occasional colic, rumblings in the abdomen, and sometimes even with continued inclination to vomit, especially after the ingestion of food. At the end of the attack, or after it, there are in many patients very distressing cramps in the calves. I have also seen moderate cooling of the extremities. I myself almost entirely lost my voice for twenty-four hours after a violent attack of this kind, and only perfectly recovered it again after several days. I have noticed also considerable reduction in the quantity of urea for several days after an attack, as well as the temporary occurrence of albumen and casts in the very scanty dark urine Catarrh of the stomach not infrequently interferes with convalescence, and errors in diet may even lead to a relapse. In other cases, temporary typhoid symptoms manifest themselves, such as headache, vertigo, roaring in the ears, cloudiness of vision, great debility sopor etc. Recovery may take place, therefore, in a few days, but it is often not perfect for one or two weeks Cholerine may be fatal to aged persons."

It will be gathered from the above description that, in contrast to choleraic diarrhoea, (*a*) the manifestations of cholerine are often marked enough to induce the patients to seek medical aid and (*b*) the symptoms and signs of this type are as a rule also characteristic enough to render the patients suspect for cholera, at least during epidemics, and thus to call for a bacteriological examination of their stools Though transitory forms exist, as a rule it is not difficult to differentiate between cholerine and cholera gravis on clinical grounds because the marked dehydration, the profound collapse and the anuria characteristic of typically severe attacks of the disease are absent in its mitigated type

Though but little information is available regarding the incidence rate of cholerine, it would appear that on the whole this form, while by no means rare is less frequent than either choleraic diarrhoea or cholera gravis

Cholera Gravis

Mode of onset

The question whether the onset of severe cholera attacks is abrupt or whether premonitory signs especially a prodromal diarrhoea, usher in the attacks, has been the subject of much debate Some of the early European observers, e g. Froriep (1832) and Géry (1868) considered a prodromal diarrhoea to be extremely rare Similarly, Macnamara (1876) in India *looked upon it as an exception rather than the rule Other authors on the* contrary asserted the frequency of this and other premonitory signs during the incubation stage of severe cholera. Thus Griesinger (1857) maintained *that a prodromal diarrhoea was present in four fifths of the cholera patients,* lasting sometimes only a few hours, in others several weeks most often 1 3 days. Lebert (1874) stated in this connexion that the frequency of this sign was apt to vary in different outbreaks while he had observed it almost invariably in Paris, he missed a prodromal diarrhoea in one-third of the cholera patients seen by him at Zürich In contrast to Griesinger who stated that an abrupt onset of cholera gravis often foreshadowed the deve lopment of particularly severe attacks, Lebert asserted that

"An attack of cholera in which premonitory diarrhoea is absent is by no means worse on this account, nor has it a less tendency to recovery than one in which it is present."

Evaluating the above statements as to a frequency of prodromal diarrhoea made before the discovery of the *V cholerae* one must naturally ask whether a causal *(propter hoc)* or merely a *post hoc* relation existed between this premonitory sign and the subsequent cholera attacks. Several of the early observers, claiming that a transition of choleraic diarrhoea into the severe form of the disease was frequent, felt convinced that such a causal relation existed. It is noteworthy however that this assumption was not supported by actual observations Thus Sticker (1912) quoting the 1850 report of the British General Board of Health, stated that

"In England there were in the year 1848 per 100 cholera sufferers 600–4000 patients with diarrhoea. The transition of the diarrhoea into a fatal cholera attack occurred in the presence of a careful mode of life and medical treatment once only among 160–200 diarrhoea cases, so that the mortality of those who developed diarrhoea was about 1 200. If the diarrhoea was neglected, it led far more often to severe attacks and 50 of 100 died." [Trans.]

These and similar observations strongly suggest that often no direct causal connexion existed between the so-called prodromal diarrhoea and the subsequent cholera attacks Nevertheless the gastro-intestinal distur

bances, though due to unspecific causes, were apt to be of importance in so far as they created a *locus minoris resistentiae* facilitating the subsequent infection with *V cholerae* Support for this assumption was furnished by observations made with the aid of bacteriological examinations Thus Rumpel (1894) stated that

"During the winter epidemic of 1892 93 we had noted the fact that in none of the 30 patients affected with choleraic diarrhoea and cholerine did the serious cholera stage follow that consequently in none of these cases had we to do with a *prodromal diarrhoea* Since in the only case of prodromal diarrhoea the bacteriological examination of which had been possible no comma bacilli could be found, we assumed that the appearance of viable cholera vibrios in the stool, without the development of the serious symptoms of cholera, was due to a personal immunity of the individuals in question. Though in the present epidemic we demonstrated the presence of comma bacilli in two instances of prodromal diarrhoea, the above assumption appears to be valid in general, because in none of the above-mentioned 69 patients [i.e., 9 carriers without clinical signs, 25 patients with choleraic diarrhoea and 35 cholerine patients] did a severe cholera attack follow" [Trans.]

The widespread and often panicky fear created by the early manifestations of cholera in Europe and the well meant but rather unpropitious attempts to avert the danger of infection by changes in the diet mentioned above on the one hand, and the great efforts made to detect all possible cholera sufferers, on the other hand, probably explain why in some of the early European outbreaks such enormous numbers of diarrhoea patients were encountered. It is obvious that most of the latter could not have been cholera affected *ab initio* and even had no occasion for a subsequent infection with *V cholerae* because otherwise the disease would have been far more rampant than it actually was

Certain it is that modern workers who have had opportunities for ample observations of their own have been practically unanimous in stating that the presence of a prodromal diarrhoea in cholera gravis was infrequent, if not exceptional. This is well exemplified by the studies made, in the case of 687 and 689 cholera patients respectively by Tao and co-workers (1948) and by El-Ramli (1948) The first mentioned authors recorded that

"The onset of illness was abrupt in most of the cases. Premonitory symptoms were rarely encountered."

Similarly El Ramli declared that

"The onset is almost always sudden with diarrhoea or vomiting and may occur at any time, even while the patient is asleep"

Stage of evacuation

Diarrhoea and vomiting

The sequence in which diarrhoea and vomiting, the most outstanding signs manifesting the first stage of severe cholera attacks, make their appearance, varies to some extent. Most observers maintain that diarrhoea is the

first sign, followed more or less rapidly by vomiting and other manifestations of the disease. However it is sometimes stated that vomiting may commence not only simultaneously with but even before onset of the bowel evacuations. Thus, El-Ramli (1948) recently recorded that in a series of 212 cholera patients diarrhoea preceded vomiting only 86 times and followed it 43 times, whereas in 83 of the sufferers both made a simultaneous appearance. He also drew attention to a few instances in which the patients were giddy, or nauseated and giddy before the evacuations from the gastro-intestinal tract commenced. Still, while no hard and fast rule can thus be laid down, it may be maintained that diarrhoea usually ushers in the evacuation stage of cholera gravis.

It is further important to note that diarrhoea is comparatively a more constant and persistent sign of severe cholera attacks than vomiting. Thus, according to Tao and colleagues (1948) diarrhoea was present in all their 687 patients and vomiting in only 86%. Similarly El Ramli (1948) recorded that, in a series of 689 cholera patients, diarrhoea was observed in 95%, but was accompanied by vomiting in 85% only the presence of the latter alone was exceptional. Interesting data of Tao and co-workers regarding the duration of these two types of evacuation may be tabulated as follows

Days	Duration of diarrhoea		Duration of vomiting	
	number	%	number	%
1	61	8.9	368	62.3
2	134	19.5	114	19.3
3	122	17 7	48	8 1
4	120	17.3		
5 or more	250	36.6		
4 / 5 or more (combined)			61	10.3
Total	687	100.0	591	100 0

It will be noted that in a considerable majority of the patients diarrhoea was of so long a duration as to outlast the evacuation stage. Vomiting, on the contrary continued in more than half of the sufferers for one day only and in 81 6% not beyond two days.

Less detailed data furnished by El Ramli (1948) showed similarly that (*a*) the duration of the diarrhoea varied from 12 hours to 15 days with an average of 4 6 days, whereas (*b*) vomiting lasted 1-8 days with a mean of 2.8 days 82% of the patients had ceased to vomit within the first 4 days of illness, at which time diarrhoea persisted in 79%.

The character of the evacuations voided during the first stage of cholera gravis undergoes typical changes After an initial period, during which fluid but still bilious stools are excreted these become acholic and finally assume the notorious rice watery appearance consisting of a colourless fluid in which colourless or whitish floccules are suspended Describing these evacuations, Liebermeister (1896) stated

"The rice watery stools have a faint insipid [*faden*] odour which has been likened by some to that of sperm, whereas one misses the proper faecal odour The reaction is mostly alkaline, sometimes neutral The mucous floccules which render the fluid turbid, contain sporadic leucocytes and fat droplets, further intestinal epithelia, which partly still form smaller lamellae and represent the cast-off cover of the intestinal villi or the inner layer of the tubular intestinal glands, but are partly changed into detritus or a formless mucoid mass finally they contain the specific cholera bacilli, often in a slight, often in a large, amount, sometimes even so abundantly as to represent nearly pure cultures. Not rarely one also finds erythrocytes, and if these are present in a large amount, the fluid resembles a meat infusion [*fleischwasserähnlich*]." [Trans.]

As with the stools, the vomited masses consist first of food remnants, then of uncharacteristic fluid masses, but finally assume an aspect quite similar to that of the rice-watery stools Like the latter, the vomits of cholera patients may show a reddish tint due to the presence of erythrocytes.

To judge from the rather discrepant statements made in this respect, the frequency of diarrhoea and vomiting during the first stage of cholera gravis is apt to vary to a considerable extent Thus El Ramli, confirming the statements of many earlier workers noted that

"The frequency of defaecation varies. It may be as many as 20 or 25 times in the first twelve hours or it may be 2 to 3 times in the 24 hours."

Statistics on this point collected by Tao and co-workers indicated that more than half of their patients (56 7%) had more than 10 stools per day Vomiting was comparatively less frequent.

To what extent the series of the workers quoted above included less severely affected patients is difficult to decide It is certain that the numerous evacuations taking place in the typically severe form of cholera gravis lead to a massive, sometimes even an almost incredibly excessive loss of body fluids Dealing with this plight of the sufferers, Corbyn wrote in 1832

"To those who have not seen persons labouring under this disease it will not be easy to convey an idea of the enormous amount of these discharges. It seemed as if the whole fluids of the body would have been insufficient for their supply The evacuations were sometimes poured forth in a continuous stream, as if from a sluice at others ejected in small volumes, as if from a syringe, by the violent action of the stomach and rectum."

Besides, like Corbyn becoming impressed by the copiousness of the evacuations in the first stage of cholera gravis, most subsequent observers also stressed that the stools and vomits were passed "rapidly without any hindrance, as if voided from a tube" (Griesinger 1857) and that defaecation was never followed by any tenesmus The manner in which the evacuations take place soon after the onset of the disease was well depicted by Wardener (1946) thus

"The evacuations were separated by intervals of an hour or more and were often followed by a sense of relief According to the severity of the attack, vomiting would follow a few hours later It was rarely frequent, but was remarkable for its lack of effort.

Pints of fluid gushed out from a patient's mouth without any straining or apparent distress. I was once questioning a patient suspected of cholera who was standing up apparently fit and resenting the imputation. Up to that time he had had only one rather loose and copious motion. As he was talking he suddenly turned his head aside, brought up two pints of clear fluid, and resumed the conversation as if nothing had happened. Within two hours he was in the algid stage."

The question whether the violent voiding of stools and vomits, characteristic of the evacuation stage of cholera gravis, is accompanied by abdominal pains, has been differently answered, a few authors asserting that such colic like pains are frequently met with, while many other observers testify to the usual absence of this symptom. There can be no doubt that the presence of intra abdominal pains is often merely apparent, the patients actually suffering from muscular cramps which, as will be discussed soon, may involve the musculature of the abdominal wall Corbyn (1832) for instance, seems to have referred to the presence of such cramps when stating that

"The patient always complains of pain across the abdomen, which is generally sore to the touch and swelled from the scrobiculus cordis to the pubes, sometimes hard and knotted, and drawn back towards the spine."

At the same time however colic-like abdominal pains may be met with in a minority of the sufferers Thus El-Ramli (1948) recorded that in 29 out of 689 cholera patients " the diarrhoea or vomiting was accompanied or preceded by colic " while Tao and co-workers (1948) even noted the presence of abdominal pain in 117 (17 0%) of their 687 patients.

Rogers (1921) maintained that severe abdominal pains associated with a marked and persistent burning sensation in the stomach were apt to be present particularly in patients voiding pink, blood-containing stools, and added that such sufferers invariably succumbed While instances of this kind are undoubtedly rather rare the frequent presence of a burning sensation in the stomach of the sufferers is generally admitted. Quite possibly however this symptom is often related to the burning thirst by which severely attacked cholera patients are usually tormented.

Muscular cramps

The appearance of muscular cramps is a further feature usually becoming distressingly manifest in the evacuation stage of cholera. Griesinger (1857) furnishing in this as in many other respects a classical description, stated that these cramps

"begin as a rule with the first copious rice-watery dejections, with the deterioration of the pulse, but very rarely before the characteristic evacuations (when a transudation into the intestinal lumen may already have taken place). Invariably the cramps are tonic, first of all frequent in the muscles of the calves, next in the toes, arms, fingers hands and thighs, rarely and mostly in very severe attacks only in the face, the jaw the rectus abdominis, the thorax musculature general or tetanic forms almost never

occur The cramps are often heralded by twinges or tickling in the muscles concerned and then set in suddenly either spontaneously or after minimal movements, commonly last a few minutes, then cease for a short time and recur They are painful, sometimes most markedly so during them the muscles are felt to be hard and rigid " [Trans.]

The only objection to this description is that cramps of the abdominal musculature are by no means as rare as Griesinger indicated. As noted above, involvement of this group of muscles may lead to the impression that the patients suffer from intra abdominal pains. More ominously still the presence of cramps in the muscles of the abdominal wall may be co-responsible for the abortions or miscarriages often occurring in cholera affected pregnant women, though presumably cramps of the uterine musculature play a still more important role in this respect (Strong, 1944)

Griesinger pointed out that the muscular cramps were apt to be more marked in young and vigorous individuals contracting cholera than in persons who had been in a weakened condition thus, as had been noted by early observers in India (see also Rogers, 1921) the muscular Europeans suffered far more in this respect than the Indians However muscular cramps were usually pronounced in the male cholera patients recently met with in China, most of whom came from the labourer or small farmer classes according to Tao and co-workers, such cramps were present in 60 4% of their series of 687 patients (431 males and 256 females) almost 65% of whom were from 11 to 40 years old.

Much dissension exists regarding the cause of the muscular cramps described above. Griesinger (1857) after reviewing the divergent opinions held in this respect by earlier writers, considered as most likely a derivation of the cramps

" from anaemia and blood concentration (Polunin [1849] and others) by analogy with the cramps following considerable blood losses, and I would hold it more probable that this factor exerts a local action in the muscles rather than one in the nerve centres. The rapid diminution of the blood volume in the muscles owing to the deficient [*stockenden*] arterial inflow seems to produce the phenomenon in the same way as one sometimes observes similar painful muscular cramps in arterial embolism." [Trans.]

At the same time however, Griesinger did not rashly dismiss the postulations of some of the early cholera workers that physico-chemical changes in the muscles, leading to an abnormal metabolism might also play a role in the production of the cramps.

Modern observers are partly inclined to lay emphasis upon such metabolic changes, incriminating in particular the rapid loss of body fluids in the evacuations, while some are led to assume that both locally acting circulatory and metabolic factors are responsible for the causation of the muscular cramps

However in view of the fact that, not rarely the muscular cramps appear quite early before signs of circulatory failure have become pronounced, this failure seems of much less importance in causing the cramps

Pints of fluid gushed out from a patient's mouth without any straining or apparent distress. I was once questioning a patient suspected of cholera who was standing up apparently fit and resenting the imputation. Up to that time he had had only one rather loose and copious motion. As he was talking he suddenly turned his head aside, brought up two pints of clear fluid, and resumed the conversation as if nothing had happened. Within two hours he was in the algid stage."

The question whether the violent voiding of stools and vomits, characteristic of the evacuation stage of cholera gravis, is accompanied by abdominal pains, has been differently answered, a few authors asserting that such colic like pains are frequently met with, while many other observers testify to the usual absence of this symptom. There can be no doubt that the presence of intra-abdominal pains is often merely apparent, the patients actually suffering from muscular cramps which, as will be discussed soon, may involve the musculature of the abdominal wall. Corbyn (1832) for instance, seems to have referred to the presence of such cramps when stating that

"The patient always complains of pain across the abdomen, which is generally sore to the touch, and swelled from the scrobiculus cordis to the pubes sometimes hard and knotted, and drawn back towards the spine."

At the same time, however colic like abdominal pains may be met with in a minority of the sufferers Thus El-Ramli (1948) recorded that in 29 out of 689 cholera patients " the diarrhoea or vomiting was accompanied or preceded by colic " while Tao and co-workers (1948) even noted the presence of abdominal pain in 117 (17 0%) of their 687 patients.

Rogers (1921) maintained that severe abdominal pains associated with a marked and persistent burning sensation in the stomach were apt to be present particularly in patients voiding pink, blood-containing stools, and added that such sufferers invariably succumbed While instances of this kind are undoubtedly rather rare, the frequent presence of a burning sensation in the stomach of the sufferers is generally admitted. Quite possibly however this symptom is often related to the burning thirst by which severely attacked cholera patients are usually tormented.

Muscular cramps

The appearance of muscular cramps is a further feature usually becoming distressingly manifest in the evacuation stage of cholera. Griesinger (1857) furnishing in this as in many other respects a classical description, stated that these cramps

"begin as a rule with the first copious rice-watery dejections, with the deterioration of the pulse, but very rarely before the characteristic evacuations (when a transudation into the intestinal lumen may already have taken place) Invariably the cramps are tonic, first of all frequent in the muscles of the calves, next in the toes, arms, fingers hands and thighs, rarely and mostly in very severe attacks only in the face, the jaw the rectus abdominis, the thorax musculature general or tetanic forms almost never

that *V cholerae* was responsible for these attacks of sthenic disease Likewise one cannot agree with Sticker s assertion that at the onset of the usual asthenic form of cholera

" some indications [*Andeutungen*] of an increased activity of the heart and the other organs are almost invariably found to be present If only the physician arrives early enough, and that the signs of deterioration [*Unterliegens*] are really peculiar only to the advanced, developed disease " [Trans.]

For it is generally agreed that from the first, signs of exhaustion and incipient collapse dominate the clinical picture Griesinger (1857) for instance, stating that the main general disturbances making an early appearance consist

" of dizziness and a feeling of anxiety which appear with the earliest deterioration of the pulse, as it would seem, with the first copious bowel transudation. These disturbances are accompanied by a change of the facies, disturbed features, great weakness, ringing in the ears, etc., and may if they appear rapidly lead to fainting fits." [Trans.]

The anxiety initially betrayed by the patients is not, as a rule very great, most of them exhibiting from the first " a certain indifference " to their distressing condition (Lebert, 1874) Wardener (1946) who was able continuously to observe the onset of cholera among his fellow prisoners of war even maintained that at that time apathy was a most striking feature

While anorexia becomes manifest immediately and may be accompanied by a bitter or pasty taste in the mouth thirst appears soon and is apt to become so distressing that the patients are compelled to drink continuously even though each draught induces further vomiting.

A state of imminent collapse is indicated by (*a*) signs of incipient circulatory failure the quality of the pulse showing a progressive deterioration while the pulse rate becomes more or less, but according to Lebert (1874) never more than moderately accelerated and (*b*) a drop of the body temperature, which as claimed by Liebermeister (1896) becomes somewhat lowered even in the rectum during the evacuation stage of cholera.

Though, as noted in Chapter 8 it is difficult to gauge the urine secretion owing to the violent purging, there can be no doubt that oliguria commences soon and that anuria may occasionally follow even before the algid stage has been reached

According to some observers hiccough may appear or even become continuous in the early stage of cholera gravis. It is, however curious to note that in China at least this distressing sign was infrequent not only then but also in the later stages of the disease, Tao and co-authors (1948) for instance, noting the presence of hiccough in only 41 (i e about 6%) of their 687 patients.

Duration of the evacuation stage

Standing, as Napier (1946) aptly put it, in an inverse relation to the severity of the symptoms the duration of the evacuation period may show

than the often quite rapidly evolving loss of body fluids. Dealing with this problem some authors, e g. Shattuck (1951) suggested with much reason that the etiology of the cramps met in cholera might be identical with that of heat cramps (often called stokers cramps) which, as Cecil & Loeb (1955) recently put it, are

" most commonly seen among manual workers in hot environments whose body fluids have been depleted of sodium chloride by unreplaced heavy losses in sweat "

It would appear that the cramps in cholera as well as the heat cramps are amenable to treatment with sodium chloride. But, inasmuch as in cholera therapeutic use is invariably made of saline *infusions*, one might argue that it is the replacement of the lost water because it restores the blood volume and therefore the circulation, which accounts for the successful treatment of the cramps Since, however according to Rogers (1921) these often disappear

" after only a pint or so has been run in, and the patient commonly falls asleep before the process is concluded "

the re-establisment of the electrolyte balance through the administration of sodium chloride probably plays a more important role than the restoration of the blood volume.

Other early signs

If it is borne in mind that the evacuation stage, ushering in the attacks of cholera gravis instead of being static for any length of time, is really a phase of incessant and often even most rapid deterioration in the condition of the patients, the discrepant descriptions given by different observers of the early symptoms and signs are easily understood Some authors consider that apart from the triad of diarrhoea, vomiting and muscular cramps described, above, only a quickly developing exhaustion of the sufferers is characteristic of the initial stage of the disease. Others, paying attention to the symptoms and signs which though becoming maximal in the following algid stage, begin to make their appearance in the evacuation phase, give descriptions fitting the former rather than the latter While, in order to avoid repetition, it is not proposed to adopt this method, the following points deserve attention at the present juncture

Dealing with the onset of " major " cholera attacks, Sticker (1912) felt entitled to distinguish a " sthenic " form from the usual asthenic form of the disease This was characterized by severe heartburn most violent pains in the intestines, tenesmus, fever and signs of congestion similar to those seen in acute lead poisoning, in addition to diarrhoea and vomiting. However though Sticker claimed that this type of cholera, in which recovery was usual, had been seen by early observers in India (more frequently among Europeans) as well as in Europe, it was never referred to after the discovery of *V cholerae* except by himself It is rather unlikely therefore,

cholerale expression—features pinched, skin drawn, eyeballs sunken and surrounded by a dark areola, lids half-closed, pupils contracted, mouth open, teeth covered with sordes, tongue cold, face apathetic. The general surface is cyanotic and clammy or bedewed with cold sweat the fingers and toes are wrinkled. There is great restlessness and profound debility The intelligence is clouded, the senses impaired, the muscular power diminished in some cases sense and sensibility and capacity of movement are retained in others coma or a semi-comatose state exists. The voice is husky and feeble or the patient can speak only in faint whispers. Thirst is imperative and a feeling of coldness is felt. The urine is suppressed the bladder is generally emptied in the preceding stage, and no further accumulation of urine takes place. The temperature of the surface and mouth is greatly and in fatal cases increasingly depressed, and may fall below 90°F [32.2 C] the temperature of the axilla is higher but below normal, readings of 95 [35 C] to 97°F [36.1 C] being not uncommon in this stage the rectal temperature may be slightly subnormal or normal, but in time it shews a tendency to rise above the normal."

Fully adequate though the above description is in most respects, it has to be pointed out that in the experience of most observers the intelligence of the algid cholera patients was not clouded. Thus Tao and colleagues (1948) stressed that "in spite of the severe prostration preservation of a clear mentality was a striking feature" However, as Liebermeister (1896) put it, the sufferers are usually so apathetic as to evince little interest either in their environment or in their own condition. Probably therefore Liebermeister was right when asserting that

"In general the subjective sensation of being ill [*Krankheitsgefühl*] is usually much slighter than one would expect from the severe objective signs." [Trans.]

Supplementary information on the symptomatology of the algid stage may be classified as follows

Dehydration

According to El Ramli (1948) the various clinical signs of dehydration were met with in the following order of frequency

"Weak or imperceptible pulse, low or not measurable blood pressure, cold skin, sunken eyes, cyanosis of nails and lips, diminished elasticity of the skin, oliguria, anuria, husky feeble voice, dry tongue, thirst, pinched nose, anxious look, washerwoman's hand, restlessness, muscular cramps, dyspnoea, pericardial rub and oppression in the chest."

It is noteworthy however that Tao and colleagues (1948) who saw a far higher percentage of seriously affected cholera patients than El Ramli enumerated intense thirst (met with in 96 1% of their 687 patients) and signs of skin dehydration characterized by loss of elasticity and wrinkling, the latter especially over the dorsum of the fingers (present in 81.2%) among the most frequent signs of dehydration Cyanosis was found to be present in but 35 5% of the sufferers

As can be gathered from El Ramli's study, the relation between the severity of the cholera attacks and the degree of dehydration was not quite constant some of the patients who appeared to have been mildly affected,

considerable variations Rapidly following evacuations may produce the dehydration and circulatory failure characteristic of the algid stage within as little as two hours while, on the other hand, it may take 12 hours or even more before that stage has become fully developed To what extent differences in the severity of the infection and in the individual susceptibility of the patients are responsible for these marked differences in the length of the evacuation period it is difficult to decide Presumably, however as a rule both these factors are of importance to a varying degree

Algid stage

Even the full development of the algid or as it is often called, the collapse stage of cholera gravis[1] does not necessarily lead to a disappearance of the signs predominating during the first phase of the disease described above. The muscular cramps appearing in the evacuation stage *may continue to torment the collapsed cholera patients or the cramps* may even first become manifest in the algid stage Sometimes vomiting may persist or as is occasionally the case as early as the evacuation stage may be replaced by retching. Moreover as aptly stated by Macleod (1910)

> "Liquid colourless motions may still be occasionally passed involuntarily or the presence of watery material may be detected in the intestines by palpation or succussion."

Rogers (1921) pointed out with much reason that

> "the lessened secretion by the bowel may be only due to the great failure of the circulation and concentration of the blood, and so be an unfavourable rather than a good sign. This view is borne out by the fact that intravenous injections of normal saline solution are commonly followed by renewed copious rice water stools."

Giving an excellent description of the algid stage of cholera gravis, Macleod (1910) stated that, though muscular cramps as well as lesser scale bowel evacuations might persist, their continued presence is

> "overshadowed by the evidences of failing power the pulse flickers and fails at the wrist, and is sometimes imperceptible in the brachial and almost so in the femoral arteries the rate, always accelerated, may rise to 120 or 140, or even higher The heart-sounds get less distinct, especially the first in some cases murmurs and friction sounds are detected in this stage "

"The capillary reaction" Macleod continued,

> "becomes slow and feeble, the surface gets livid respiration is quick and shallow painful and often paroxysmal dyspnoea arises, compelling the sufferer to struggle for breath the expired air is cold, and deficient in carbonic acid. The face presents the characteristic

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[1] Sticker (1912) was dissatisfied with both these designations and also pointed out that, as discussed already by Liebermeister (1896), the name of *stadium asphycticum*, used instead by some writers, since it refers merely to a state of pulselessness, was also inadequate. He recommended, therefore, the use of the term *stadium paralyticum* (already used by some earlier writers) to indicate the extreme weakness of all vital functions during the second stage of cholera gravis. Since, however, the designations of algid stage or collapse stage have been widely adopted and, moreover as defined in the large dictionaries, they have a meaning quite similar to that of *stadium paralyticum*, no cogent reason exists to give them up in favour of the latter unfamiliar designation which, in its turn, is somewhat misleading.

and increased conduction time These showed immediate improvement when potassium was administered "

As summarized in the *Tropical Diseases Bulletin* (1948) Baligh considered heart failure as the most common complication of cholera, being met with in

" (1) most of those patients dying within 24 hours of admission, as a result of sudden changes in the blood and ischaemia (2) sometimes during treatment with large transfusions given rapidly (3) occasionally during apparent convalescence as a result of toxaemia and myocarditis. Extrasystoles were quite common at this stage but corrected equilibrium of the blood soon caused them to disappear Electrocardiographic examinations confirmed that the heart may be so deranged in severe cholera as to fail at any moment during convalescence. In fact 8 apparently well patients did die suddenly at this stage in 10 to 20 days from the onset of the disease "

Godel evidently dealing with the same group of patients, stated that

" *the general aspect of the electrocardiogram, considered in its four derivations, shows* a striking similarity to acute coronary insufficiency It is the more tempting to come to this interpretation, because the heart often shows a galloping rhythm and the patient feels some retrosternal pain in spite of the prostration numbing his sensitivity" [Trans]

Godel stressed that intravenous infusion of normal saline solutions containing calcium, though it brought the blood concentration to normal did not immediately lead to changes in the electrocardiogram, which became normal only after intervals of up to 12 days. He also stated that the patients suddenly succumbing either during the acute phase of cholera (i e , on the third to fifth day after onset of the disease) or after 12 15 days in the stage of convalescence, frequently showed " salvoes " of extrasystoles a few hours before death indicating the imminence of ventricular fibrillation. In his opinion " the coronary circulation was involved in these dramatic incidents."

Respiration

Giving a description of the type of respiration met with in the algid stage of cholera similar to that of Macleod (see page 700) Griesinger was inclined to ascribe the often marked dyspnoea and sensation of oppression in the chest mainly to the sluggish and decreased blood circulation in the lungs. He added, however, that the concentrated blood had lost much of its faculty to maintain an adequate *relationship with the air oxygen* The coldness of the breath and the reduced content of the expired air in carbon dioxide were compatible with a reduced circulation and gas exchange in the lungs.

Griesinger maintained that auscultation of the lungs led to no abnormal findings Subsequent observers referred in this connexion to the occasional presence of pleural friction in extremely dehydrated cholera patients Apparently this sign is considerably rarer than the pericardial friction

their illness lasting not more than three days, were admitted in a severely dehydrated condition Among the patients classified as suffering from moderately severe cholera (i e. 4-6 days of illness with but mild, if any complications) some showed mild, but others marked signs of dehydration Further though most of the patients classified as having severe cholera attacks were on admission in a bad condition,

"some of them came to hospital in a fairly good condition with a mild or moderate dehydration and then developed severe symptoms."

Circulatory failure

Though, as has been discussed in the previous chapter the circulatory failure becoming maximal during the algid stage of cholera is primarily of an extracardiac origin nevertheless signs of a secondary involvement of the heart in this process are apt to become manifest. Griesinger (1857) noted in this respect a progressive weakening of the heart sounds, the second of which often became altogether inaudible, and also the occasional appearance of systolic murmurs He considered it possible though unlikely that the hypothetical cholera " poison " might exert an action on the heart, but ascribed far greater importance to " a kind of sympathetic action from the intestine " analogous to the depression of the cardiac activity occasionally observed in patients with strangulated hernias However in Griesinger's opinion a most important role was played also by the stagnant circulation and the resulting metabolic deficiencies in the heart muscle, which thus, like the musculature in general, became extremely weakened.

Propounding views similar to those of Griesinger Liebermeister (1896) also maintained that the cardiac weakness observed in the algid stage was " in cases with marked blood concentration partly due to a deficient nutrition of the cardiac musculature."

The rapid, though sometimes only temporary restoration of the blood circulation usually following even single saline infusions[1] leaves no room for doubt that the cardiac disturbances observed in the algid stage of cholera are as a rule of a functional nature only That, however this is not invariably the case, is proved by the occurrence of sudden deaths from heart failure in cholera convalescents.

Interesting observations have been made in this connexion with the aid of electrocardiography While Bien & Tung (1933) apparently the first to use this method for the examination of cholera patients, made no significant findings, some have been more recently recorded by Weaver et al (1948), Baligh (1948) and Godel (1948) According to Kamal Messih & Kolta (1948) electrocardiographic tracings made during the 1947 cholera outbreak in Egypt by Weaver and colleagues " showed left axis deviation

[1] It is of historical interest to note that Griesinger referred to instances of temporary restoration of the blood circulation effected in cholera patients with the aid of *blood* transfusions.

fest on the former while the cornea may appear more or less cloudy and as if covered with dust particles

Statements regarding the condition of the pupils vary As noted above (page 701) Macleod considered contraction of the pupils a characteristic of the algid stage of cholera while according to some other authors (e.g. Takano and colleagues, 1926) the pupils were dilated and reacted slowly to light. Quite probably the pupils are at first contracted, but may later become dilated in profoundly collapsed patients and particularly in those hopelessly affected

While Sticker insisted that even in the presence of most severe dehydration the tension of the eyeballs remained normally high Manson Bahr (1954) stated on the contrary that

"cataract may develop suddenly in the stage of collapse and may have a similar osmotic basis as that of diabetes. In cholera loss of fluid from the bowel may lead to osmotic dilution of the body fluids, including the aqueous, and consequent inflow of water into the lens."

Manson-Bahr added that

"The ophthalmoscopic changes consist of a wavy gridiron appearance with dark lines against a red background, possibly denoting wrinkling of the capsule."

Possibly a patient mentioned by Wardener (1946), whose eyesight became so impaired during a cholera attack that he could merely distinguish between light and dark, suffered from a condition similar to those described by Manson Bahr It has to be noted, however that this cholera patient and some others observed by Wardener who suffered to a lesser degree from dimness of vision were probably affected by pre-existing nutritional deficiencies It is certain that nowadays eye symptoms are generally infrequent among patients treated in well run cholera hospitals. Thus Tao and colleagues noted blurring of vision in but one out of 687 such sufferers.

A ringing noise in the ears, though rather more characteristic of the evacuation stage may occasionally be present during the algid phase of cholera as well Wardener (1946) noted progressive deafness in some of his patients admitted in a dehydrated condition, but, as noted above, these prisoners of war possibly suffered from nutritional deficiencies Tao and colleagues recorded ringing in the ears in 7 9% of their patients without stating at which stage of the disease this symptom was present.

Temperature

The problem of the body temperature in cholera has been exhaustively dealt with by Griesinger (1857) and later by Liebermeister (1896) and by Sticker (1912)

quite often noted, but one should not lose sight of the probability that the physicians attending cholera patients pay far more attention to an examination of the heart than to that of the lungs

It is striking to note that modern authors generally make little or no reference to the presence of a marked dyspnoea and sensation of oppression in the chest, as a rule merely stating that the respiration in the algid stage of cholera is rapid and shallow—signs produced according to Strong (1944) by a stimulation of the respiratory centres due to the deficient circulation and poor aeration of the blood It is quite possible that the descriptions of the early writers were often based on observations made on adult Europeans in whom, according to Sticker (1912) dyspnoea and a sensation of chest oppression were frequent, whereas, as this author put it,

"in the delicate bodies of the Hindus and of children the respiration remains regular gradually decreasing in force and deepness in the paralytic stage, and finally ceasing long after the heart has stopped to beat." [Trans.]

As a corollary to Sticker s contention it may be added that, so far as the experiences of the present writer go marked dyspnoea and a sensation of chest oppression were not at all conspicuous in Chinese cholera patients. Likewise, Tao and colleagues did not mention the occurrence of these signs in the 687 patients observed by them in a Shanghai cholera hospital.

Vox cholerica

As aptly stated by Sticker the appearance of the so-called vox cholerica is primarily due to a tendency of the greatly weakened cholera patients to articulate with their lips (*Mundsprache*) without any effort on the part of the larynx, so that at first, if they wish, they can raise their voice. Later however dryness of the vocal chords, muscular debility and lack of will-power combine to render the voice really quite feeble and husky or may even lead to complete aphonia. A cadaveric position of the vocal chords or paralysis of single laryngeal muscles could be present in this extreme stage.

Though often met with to a varying degree in cholera gravis, signs of a vox cholerica can remain absent in severely affected patients, particularly quite often in children Total aphonia was, according to Sticker a most infaust sign

Sensory organs

Most authorities maintain that the alterations which may but need not, become manifest in the eye during the algid stage of cholera are of a superficial nature a cessation of the tear secretion, combined with reduced lid movements and more or less pronounced lagophthalmus may lead to exsiccation of the exposed parts of the conjunctiva or the cornea. Brownish or blackish spots, surrounded by injected blood vessels may become mani-

Liebermeister maintained in the latter connexion that during the evacuation stage the rectal as well as the surface temperature was usually slightly below normal but that with the onset of the algid phase the latter continued to drop whereas the rectal temperature rose to reach sometimes 39° or 40°C. Like Griesinger he noted that such increases of the rectal temperature were particularly marked before death and that a post mortem rise of this temperature could be observed At the same time, however an abnormally low rectal temperature during the algid stage was also an unfavourable sign

Though postulating the occurrence of a "sthenic" form of cholera associated with fever Sticker (1912) admitted that

"Cholera is usually an asthenic disease in the truest sense, a disease without fever which is mostly associated *ab initio* with a drop of the body temperature." [Trans.]

However, marked differences between the surface and the rectal temperatures were apt to occur Moreover slight initial rises of the surface as well as of the rectal temperature were sometimes observed in children

In contrast to Griesinger some authors continued to assert that cholera was a febrile disease Thus, as quoted by Rogers (1921) Chevers (1883)

"described cholera as a fever in which the rise of temperature is masked during the collapse stage by the failure of the circulation through the peripheral parts, but becomes apparent during the reaction period of the disease."

Accepting Chevers's postulation Rogers (1952) referred to a patient who had manifested a variola rash during the convalescent stage of cholera, but had not shown the initial fever characteristic of smallpox during the algid stage However one must remark that such an absence of a febrile reaction to an extraneous infection does not furnish proof that invasion of the body by *V cholerae* must necessarily lead to such a reaction which is suppressed during the algid stage Generally speaking, most modern observers, in contrast to Rogers, do not share the belief of writers like Chevers that cholera is typically a febrile disease. Thus Napier (1946) stated

"Cholera has been described as a disguised febrile disease, the fever being suppressed in the collapse stage the writer questions this interpretation and doubts whether the early rise that may occur is really part of the cholera syndrome. He has seldom seen any febrile reaction in an uncomplicated case that could not be accounted for by the infusion of pyrogen-containing saline."

El Ramli (1948) dealing with the problem presently under review declared that

"The majority of our cases ran an afebrile course (65 %) When a patient was first seen, the temperature was generally subnormal or normal and *remained so*, or rose to normal and remained as such. Sometimes the temperature rose to above normal at the end of the diarrhoea stage (70 % *of the febrile cases*) In 30 % of these cases, the diarrhoea was still present, although the temperature had subsided. 12 % of the cases showed a rise of temperature on the first day of illness."

Griesinger stated that, though the lowering of the surface temperature of the peripheral parts of the body was so characteristic of cholera as to lead to the definition of the second phase of the disease as the algid (cold) stage,

"the actual temperature decrease is not as marked as it seems to the hand of the examiner be it that the moisture simultaneously present gives the impression of a marked and untoward coldness or as is indicated by the strikingly slow rise of the thermometer that there is an actual reduction of heat radiation." [Trans]

While doubting that the surface temperature of the extremities could actually sink as low as 20° 25°C, as had been claimed by some earlier observers, Griesinger considered temperature readings of 29° 31°C (i.e., about 6 -8°C below normal) as correct. The temperature in the oral cavity and that of the tongue hardly ever fell below 30°C.

As shown by its close association with the degree of cyanosis, the temperature loss in the extremities was due mainly to the circulatory failure. However the occasional occurrence of a considerable algor of the extremities in patients still having a fairly good pulse proved that an inability of the tissues to generate heat, i.e "a kind of acutest tissue inanition" also played a role.

Provided that sufficiently prolonged thermometry was resorted to the temperature of the trunk was as a rule found to be normal or but slightly lowered, measuring not less than 35°C in the axilla. As Griesinger noted, statements regarding the rectal and vaginal temperatures were discrepant, some observers recording low others markedly increased, values. High temperatures were also occasionally found in the axillary cavity However Griesinger emphasized, these observations did not indicate that cholera was an "inflammatory" or occasionally febrile disease, as had been claimed by some writers. On the contrary it appeared that, as in other infectious diseases, for instance typhoid, such high temperatures were apt to become particularly manifest immediately before death of the sufferers. Griesinger admitted that

"the cause of this pre-agonal temperature rise was unknown apparently it may involve the whole body or only the internal organs, but it has nothing to do with the prolongation of vital processes. For in some instances the temperature increase takes place only after death Moreover the dead bodies of cholera victims continue to cool with a striking slowness it would seem that the reduction of heat loss, which is striking in the patients, still continues after death." [Trans.]

In agreement with Griesinger's views, Liebermeister (1896) stated that in the algid stage of cholera

"the temperature of the peripheral parts of the body drops considerably in the closed palm it frequently reaches only 30°C or less, and the ears and tip of the nose are often still considerably cooler But the trunk of the patients feels far less cold, and the temperature in the interior of the body in spite of the low temperature of the peripheral parts, is frequently normal or even increased." [Trans.]

Stage of reaction

It is one of the most distressing features of cholera that the termination of the algid stage is by no means invariably followed by a rapid and uncomplicated restitution of the health of the patients. Quite often, on the contrary a tardy and incomplete improvement of their condition exposes them to the danger of relapses, or untoward (or as Griesinger (1857) called them "excessive") reactions may take place which threaten or frequently cut short the life of the sufferers One may thus distinguish between three main types of the reaction stage which will now be dealt with seriatim

Rapid restitution of health

The signs of uneventful and rapid recovery from the algid stage of cholera have been shortly but adequately described by Macleod (1910) thus

"The pulse returns to the wrist, feebly and fitfully at first but there is, in favourable cases, a progression in steadiness and strength. The breathing becomes easy and the patient tranquil. Blueness, coldness, shrinking and clamminess of the skin give way to roundness and warmth. Temperature is normal or slightly raised. The stomach regains its tone, and food is retained. The stools resume their proper colour some looseness may persist, but the motions are less frequent and less watery and exhibit deepening tints of grey and brown. Urine is passed, though its appearance may be delayed for many hours It is at first scanty high-coloured, of strong smell, high specific gravity albuminous and containing indican and casts then it becomes copious. Mental activity and muscular power return, and complete recovery may take place within a few days."

Imperfect and interrupted reaction

A retarded and incomplete recovery from the algid stage may as Griesinger put it, lead to "various intermediate states of an oscillatory and dangerous character" Usually incipient signs of a disappearance of the algid stage become manifest, but further progress is irregular and incomplete Thus the warmth of the body may not be uniformly restored particularly in the extremities Diarrhoea may persist the urine secretion may not be resumed or may remain insufficient. An insufficiency of the peripheral circulation may continue to be manifest. Deaths from sudden heart failure may take place even at times when the general condition of the patients seems to improve. The sufferers remain in an exhausted condition and may once or repeatedly relapse into the algid state Though uneventful recovery may take place even after repeated collapses, as a rule the patients either succumb while in an algid condition or temporarily showing signs of improvement, they may fall into an uraemic condition Uraemia may develop even if a careful watch is kept over the patients so as to deal with impending relapses.

Recording the results of clinical observations of 687 cholera patients, Tao Woo & Loh (1948) stated

"Because of the frequent occurrence of reactions to saline in the form of chills and fever it is difficult to ascertain the actual incidence of fever in this disease. Among 260 cases seen in August, when improved pyrogen-free saline was used and reactions occurred rarely slight or moderate fever (as measured by rectal thermometer) was noticed only in 31.5% or 82 cases."

Duration of the algid stage

No fully satisfactory information on the duration of the algid stage of cholera can be derived from the classical writings of authors like Griesinger (1857) Lebert (1874) and Liebermeister (1896) because (*a*) in many respects they dealt with the attack stage of the disease comprising the stage of evacuation and that of collapse instead of separately dealing with each of these phases and (*b*) they came to the conclusion that patients showing signs of profound collapse, who nowadays can quite often be cured through adequate infusion treatment, mostly died Thus Griesinger concluding his description of the "asphyctic or paralytic" phase of the cholera attack, stated that

"Only rather rarely does recovery from this condition take place in the overwhelming majority of the cases the aspect of the sufferers becomes progressively more like that of a corpse, the coldness of the skin increases, sticky sweats appear the eyes, with the eyeballs turned headwards, remain half open, the action of the heart ceases, the respiration becomes deep, sighing or stertorous, sensory functions and consciousness disappear" [Trans.]

As Griesinger added

"A large number of the patients die in this manner after the stormy manifestations of the attack have ended. Death can take place after the attack has lasted as little as two hours, very often in the course of the first day often also on the second day The attack almost never lasts longer than 24-36 hours." [Trans.]

Similarly Liebermeister added to a description of profound cholera collapse that

"Finally this death-like condition passes over into actual death, sometimes a few hours after onset of the attack, frequently in the course of the first, or at the latest within the second day In more rare cases the sufferer rallies and enters the regressive stage." [Trans.]

While stating that the length of the collapse stage could vary from a few hours to two days, Rogers (1921) emphasized that the chances for the patient deteriorated *pari passu* with the longer persistence of the algid condition. In this connexion he quoted Goodeve (1866) according to whom an uneventful convalescence could be expected if the algid stage did not last longer than 8-10 hours, but complications had to be anticipated if the patient could be brought round after having been collapsed for 18-24 hours. The danger of uraemia, in particular was the greater the longer the collapse lasted.

Fraenkel s publication these sufferers did not succumb to hyperpyrexia but to uraemia As will be further discussed below, such a combination of a febrile condition with the subsequent manifestation of uraemia has also been noted by later observers.

Modern workers while not denying the occurrence of hyperpyrexia in the reaction stage of cholera, consider it infrequent The validity of this contention is borne out by the recent observations of El Ramli (1948) and of Tao and co-workers (1948) The first mentioned worker declared that

"Very few cases had high fever and we did not encounter hyperpyrexia nor was the fever any trouble to us."

Tao and colleagues, referring to the problem of hyperpyrexia, which they classed among the complications of cholera stated that

"Transient rise in body temperature up to 39°C. or more (as measured by the rectal thermometer) following saline infusions was found in the great majority of patients especially in the first month, when the saline used was known to be not pyrogen-free Prolonged elevation of the temperature above 40°C. lasting over 12 hours, unexplained by any inflammatory complication, occurred in 4 cases, all of which were among children and proved fatal. Convulsions developed in two of them."

These observations are in agreement with the contention of Lebert (1874) that the stage of reaction was " more intense " in cholera affected children

" *Cholera typhoid* " A consideration of the voluminous literature dealing with the so-called cholera typhoid clearly shows to an unbiased observer that the altercations on this subject were due mainly to repeated attempts to make a clinical entity out of a clinical syndrome or one should rather say, syndromes characterized by the presence of a status typhosus Particularly noteworthy in this respect is the fact that some authors, and most empha tically Frerichs (1851) felt convinced that the patients exhibiting signs of a status typhosus actually suffered from post-choleraic uraemia On the other hand, even some modern observers considered the cholera typhoid a febrile affection Rogers (1921) for instance stating that

"In addition to the hyperpyrexial form of reaction, a less common typhoid-like form occurs, in which the temperature remains persistently high, even although no saline injections may have been given, such as at about 103 [F (39 4°C)] for two or more days "

Significantly Rogers added that

"these cases are very fatal and difficult to deal with, especially if accompanied by symptoms of uraemia, as is often the case."

In contrast to the above postulations, other authorities asserted that the name of cholera typhoid was used to designate different syndromes observed in the reaction period of cholera Griesinger a vigorous advocate of this concept though stressing the necessity of distinguishing between these various affections, held that for the sake of brevity the common and sympto-

Excessive reactions

Dealing with what Griesinger aptly called excessive reactions becoming manifest in the third stage of cholera gravis, modern authors refer merely to hyperpyrexia and to uraemia. However before assessing the importance of the latter symptom complex, attention has to be devoted to what earlier writers discussed voluminously under the name of cholera typhoid.

Hyperpyrexia As has been alluded to above (see the quotation from Napier 1946 on page 707) and as will be fully discussed when dealing with the treatment of cholera, accesses of fever not rarely ushered in by chills, which take place when the patients react to saline infusions, are generally due to the presence of pyrogenetic substances in the fluids used However as proved by ample descriptions of the early writers (e g Griesinger 1857 and summary by Sticker 1912) as well as by further observations, an excessive febrile reaction unconnected with infusion treatment may also occur Liebermeister (1896) briefly but adequately referred in this connexion to a " reaction fever " appearing in the third stage of cholera, which could last for one or several days, and could be associated with a frequent and dicrotic pulse headache sometimes even with somnolence and delirium—a description which tallies well with that given more elaborately by Griesinger for the excessive febrile reactions apt to become manifest in that phase of the disease

Interesting statements on hyperpyrexia in cholera were made by Rogers (1921) who maintained that

> " except occasionally in very mild cases, some rise of temperature occurs during reaction. In those recovering without transfusion it usually only reaches from 100° to 102 [F (about 37.8°–39°C)] in the axilla in native patients In Europeans this rise of temperature is still more marked, and frequently reaches a dangerous point apart altogether from the use of intravenous or subcutaneous saline."

In support of this contention Rogers stated that

> " fatal excessive febrile reaction was the most frequent cause of death in those Europeans who survived the collapse stage of cholera even when no saline injections had been given, and actual hyperpyrexia caused 28 per cent. of the deaths in the reaction period."

He felt convinced that " absorption of a fatal dose of toxins from the bowel with revival of the circulation " accounted for these deaths.

It has to be noted that observations made on European cholera patients by Rumpf & Fraenkel (1894) stood in curious contrast to Rogers experiences According to these two German workers there was not a single death among 170 patients who after a cholera attack developed fever without other complications, regardless of whether there had been only a single rise of the temperature or whether the fever had been remittent or continuous for several days. Out of 41 patients, in whom the fever had been accompanied or followed by coma, 25 died, but, as can be gathered from Rumpf &

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no doubt that the status typhosus may stand in causal connexion solely with the development of a highly febrile condition without the presence of signs of renal failure As has been noted above Rogers (1921) expressed the belief that the instances of the latter kind fell in a special category distinct from that in which a hyperpyretic condition developed in the reaction stage of cholera However there seems to be no justification for such a differentiation either from the viewpoint of symptomatology or from that of etiology

As referred to above Rogers was of the opinion that an absorption of the cholera toxins, taking place in the stage of reaction was responsible for the production of hyperpyrexia The same postulation has been made by some earlier observers e g. by Amako (1909) and Jochmann (1914)

Amako noted that the serum of five individuals, who had succumbed in the reaction stage of cholera after they had exhibited signs of a typhose condition did not exert an opsonic action but was endowed with marked bacteriolytic properties He further recorded that (*a*) in these individuals the cholera vibrios had disappeared from the dejecta simultaneously with the manifestation of the status typhosus and (*b*) at autopsy it was impossible to cultivate either *V cholerae* or other organisms (e g. *E coli* and cocci) from the intestinal contents or from the intestinal walls, even though the latter often showed evidence of necrosis.[1] Amako polemized, therefore against the postulation of writers like Kolle (1904) that the cholera typhoid was due to secondary infections with common intestinal bacteria and maintained like Rogers after him, that an action of the *V cholerae* endotoxin was responsible for the causation of this syndrome He declared in this connexion that

> "Whereas a gradual production of bacteriolysins is bound to exert a favourable influence on the course of the disease, their sudden and abundant appearance leads to a rapid lysis of the cholera bacteria As a consequence of this sudden lysis of numerous vibrios large amounts of endotoxin are liberated and these call forth the signs of severe intoxication characterizing the symptom complex of the cholera typhoid." [Trans.]

According to Jochmann (1914)

> "The cholera typhoid is a febrile state, in which, particularly a general poisoning with the cholera toxin, and especially disturbances of the sensorium, become prominent A high degree of fever replaces the subnormal temperature " [Trans.]

Baerthlein & Grünbaum (1916) objected to this contention of Jochmann For they stated,

> "In almost all cholera patients in whom we observed, together with typhoid appearances, a transition of the subnormal to the febrile temperature, we could bacteriologically demonstrate another complicating infection, a mixed infection, e.g., typhoid fever with the aid of blood-bile cultivation, or malaria or gonorrhoeic cystitis." [Trans.]

[1] It is interesting to note in this connexion that an absence of *V cholerae* in smears prepared from the intestinal contents of cholera victims who had succumbed in a typhose condition, had already been noted in 1846 by Weisser & Frank

matically adequate designation of cholera typhoid might nevertheless be retained He estimated that signs of this condition becoming manifest in a majority of the patients who had passed through a stage of "asphyxia" (i.e., profound collapse) appeared in one-quarter of all those suffering from true cholera. Though occasionally developing as early as two days after onset of the disease, signs of the typhose condition usually became noticeable after an illness lasting three to four days.

Expressing agreement with these statements, Liebermeister (1896) gave the following masterly description of the cholera typhoid

"The name of cholera typhoid is given to all cases in which a status typhosus develops. This consists of general debility and exhaustion with special impairment of the psychic functions. Some patients are apathetic, drowsy speak stammeringly complain of headache occasionally there is vomiting or also singultus. In others the sensorium is still more disturbed, delirium appears which is mostly quiet, with muttering, but sometimes more active. Or there is a state of drowsiness, and this may be exacerbated to fully developed coma, the unconscious patient lying with half-closed eyes, injected and mucus-covered conjunctivae, and a diminished reaction of the pupils In some cases cramps are seen in various muscle groups or more rarely general convulsions, followed by coma, make an appearance. The tongue and lips are dry and cracked, and often covered with a sooty the so-called fuliginous, layer Often râles can be heard in the lungs. In some cases there is fever with a considerable increase of the temperature, rapid and dicrotic pulse, accelerated respiration, and reddened face. In other patients the body temperature remains about normal or even abnormally low throughout the persistence of the condition or during its later period, the pulse being not rapid. Urine secretion is mostly scanty the urine is albuminous, and often contains abnormal formative elements. The spleen is but rarely enlarged." [Trans.]

As will be gathered from this classical description the patients labelled as suffering from cholera typhoid fall, as far as their temperature is concerned into three groups—namely (*a*) one in which fever remains absent (*b*) a second, in which the initially increased temperature later becomes normal and (*c*) a third, in which considerable fever and the signs usually accompanying an elevation of the body temperature persist.

As was conceded by most of the early writers and is now generally admitted, it is certain that the patients of the first group who show the signs common to what has been called cholera typhoid but no fever actually suffer from post-choleraic uraemia. Further there can be little doubt that in those who are first feverish but then became afebrile uraemia became manifest in the course of an inflammatory process Such a combination which, as stated above, was considered frequent by Rogers (1921) was also noted by earlier observers. Thus Macleod (1910) in the course of a discussion of the "typhoid" state in cholera, declared that

"There are cases which seem to occupy an intermediate position between the hyperthermic and the typhoid cases In these mild fever of an intermittent or remittent form complicates and delays recovery"

However though it is certain that uraemia plays a preponderantly important role in the causation of the so-called cholera typhoid, there can be

Describing the clinical manifestations of post-choleraic uraemia, Griesinger stated that

" as a rule the patients fall into a state characterized by a dull drowsy facial expression increasing stupor amounting to sopor but also with intercurrent excitement and delirium, slow and deep respiration with long intervals, slow pulse, and no appreciable increase of the surface temperature (hardly any or no fever) the gaze becomes staring, the pupils immotile the conjunctiva often shows an abundant secretion of mucus the tongue tends to become dry is red or shows a brownish coating the cheeks are often red the head is hot the extremities are cool and, rarely exanthemata develop diarrhoea usually persists vomiting or singultus may be present the skin is often covered with an oily sticky sweat, which leaves a residue of urea crystals In very rare instances general convulsions appear mostly general paralysis develops with the increasing sopor " [Trans.]

Giving a similar though less exhaustive description, Macleod (1910) pointed out with great reason that the looseness of the bowels met with in part of the uraemic patients was apt to be beneficial and therefore should not be checked [1] He added that the condition of the sufferers might be aggravated by a suppression of the secretion or discharge of the bile (" cholo-uraemia " according to Chevers 1883)

The following figures may be quoted to illustrate the incidence of post choleraic uraemia

Rumpf & Fraenkel (1894) stated that " coma without fever " due presumably to uraemia, was present in 183 out of about 700 cholera patients, i.e. in about 26% Fully reliable records by Nichols & Andrews (1909) showed an occurrence of uraemia in 73 (15 7%) of 466 hospitalized cholera patients. Hence features of this condition became manifest in almost one-quarter (24 4%) of the 299 sufferers who survived the collapse stage. Figures submitted by Rogers (1921) were as follows

Period	*Method of treatment*	*Total cholera deaths*		*Uraemic deaths*	
		number	%	*number*	%
1912 14	Hypertonic saline and permanganate	152	25.6	66	11 1*
1915-17	Alkaline saline solution	115	19 7	19	3.25

* As against 13.2 % in a group of patients admitted in 1907 and not treated with hypertonic saline.

As with Rogers latest figures, Tao & colleagues (1948) reported 27 instances of uraemia in their 687 cholera patients, i e., 3 9%. They added that

" Of the 27 cases of uraemia, 8 died, giving a mortality of around 30 %. Symptoms of uraemia might start at any time from the second to the sixth day of illness. Among the cases that recovered the symptoms usually lasted from 3 to 5 days, although the longest duration was 7 days. Of the fatal cases, 7 died within 3 days and only 1 died 5 days after the onset of symptoms."

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[1] According to Rogers (1921), the same advice had already been given by Goodeve (1866).

The two workers were of the opinion that one should speak of the presence of a cholera typhoid only when a status typhosus without fever became manifest.

When trying to assess the comparative validity of the divergent postulations quoted above, one must unhesitatingly express agreement with the views of Kolle and Baerthlein & Grünbaum.

For, even if one could believe that a sudden and massive absorption of the *V cholerae* endotoxin takes place in the reaction stage of cholera, it is inconceivable that this should produce high fever and not the marked drop of the body temperature observed in experimental animals to which the endotoxin is administered

Uraemia Dealing with the problem of post-choleraic uraemia, Griesinger (1857) maintained that

"strictly speaking one should ascribe to uraemia only those cases in which the urine secretion has not become established or has soon ceased again, or in which, at least, only very little urine of a very slight specific gravity and high albumin content is voided." [Trans]

Adequate though this statement is in general, it must be realized that oliguria and anuria, followed by uraemia, may develop once more in cholera patients whose urine secretion had become initially re-established. Wardener (1946) *referred in this connexion to two patients in whom uraemia became* manifest after they seemed to have recovered from cholera attacks and had voided apparently adequate quantities of urine for three weeks. While instances of this kind are certainly exceptional, shorter periods of a more or less restored renal function may not infrequently be observed. Henderson & Seneca (1951) aptly describing the curious state of imbalance which may thus exist, stated that

"Sometimes the return of kidney function is intermittent and finally unsuccessful As in other diseases with renal insufficiency the blood pressure rises as if to bring about adequate filtration by force in cholera, this will occur as well as the restored circulation can permit. Some nitrogen is thus cleared, but the blood pressure may fall off again, and anuria may return. See-sawing of this kind may continue, and the patient may die, or the quantity of urine may increase progressively albumin become a little less, and azotemia steadily diminish, until an ample flow of urine makes recovery a certainty"

It has to be added that Rumpf & Fraenkel (1894) referred to a few cholera patients in whom a fatal comatous condition developed at a time when the urine secretion had become restored or even abundant. The two workers claimed, therefore that lack of urine secretion could not be the cause of the comatose process met with in cholera. But while, as has been discussed in the previous chapter the causes of post-choleraic uraemia are complex, it is certain that almost invariably its manifestation is preceded by and associated with, marked oliguria or often even anuria. The claims made by Rumpf & Fraenkel must, therefore, be interpreted with caution

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Complications

General considerations

Etiologically the complications met with in individuals who are recovering or who have recovered from cholera fall into two categories (*a*) complications spontaneously arising either as immediate sequelae of the choleraic process or owing to the presence of secondary infections or other concomitant morbid processes, and (*b*) complications whose cause lies in inadequacies of the infusion treatment (to be dealt with in a later part of this chapter)

Macleod (1910) made an interesting but (in the light of further information) not invariably adequate attempt to classify the complications spontaneously arising into the following three groups

(1) Complications of a *functional* character comprising conditions such as anaemia, debility nervous depression, jaundice, gastric irritability persistent hiccough insomnia, dementia paraplegia, anasarca, irregularity of bowels, chronic diarrhoea, and abortion (or miscarriage) in pregnant women.

(2) Complications of an *inflammatory* nature including oedema of the lungs (?), bronchitis, pneumonia, pleurisy meningitis, conjunctivitis arthritis, parotitis and dysentery [1]

(3) *Destructive* processes such as ulcerations of the cornea, bed sores, gangrene of the nose, ears penis, and scrotum or more rarely of the fingers and toes

It is consoling to note that nowadays owing to prompt and adequate treatment and proper general management of cholera patients, several of the complications enumerated by Macleod or other early writers have ordinarily become exceptional or are even no longer encountered at all This is particularly true of " destructive " processes such as gangrene and bed-sores That, however if it cannot be checked, cholera may still lead to such ravages, is exemplified by observations made by Wardener (1946) in a group of prisoners of war who lived and whose ailments had to be treated, under most distressing conditions. As he recorded,

> " Large, painless, black and rapidly growing areas appeared at all pressure points, mainly across the sacrum and upper back. In 24 hours an area might be formed 3-4 [inches (7.5-10 cm)] in diameter After two or three days the gangrenous areas would begin to separate. In a few cases the whole patch would slough, but the patient always died before epithelisation. The pain attending the separation of the slough, the huge raw weeping areas which followed, the inadequacy of dressings, of nursing and comfort, all combined rapidly to exhaust the patient. In others there was no reaction the gangrenous area remained, becoming more extensive until death."

[1] Macleod's statement that " dropsy has been described as a result of consecutive nephritis can no longer be accepted, because in the unanimous opinion of modern observers chronic nephritic processes never develop as the result of cholera

Duration of the reaction stage

The duration of the reaction stage depends upon the absence or presence not only of the "excessive" reactions described above but also of the complications dealt with below However if such untoward deviations from the normal course of the disease remain absent, recovery of the patients progresses rapidly to become complete within a week. Similarly to numerous statements previously made in this respect Tao and co-workers (1948) reported that

"In uncomplicated cases the average duration of illness was 4 to 5 days, although in quite a number of cases diarrhea persisted for a week or longer With the supervention of uraemia as a late complication, the course of illness became prolonged to an average of 7 to 8 days."

It is important to add that the frequent continuance of diarrhoea referred to above did not depend upon the persistence of *V cholerae* in the stools. For as successive bacteriological examinations of samples collected almost daily from 218 patients showed, in almost 90% the cultures became negative for cholera vibrios on or before the sixth day of illness, while in no instance were positive findings obtained for more than eight days after onset of the disease

Relapses

It has been discussed above that an initially favourable reaction to severe cholera attacks may be of a temporary character the patients remaining, as it were in a state of imbalance during which relapses into the algid condition may occur once or even several times. Some workers, like Griesinger (1857) Macleod (1910) and Sticker (1912) also stated that after a temporary amelioration there may be a reappearance of purging and vomiting, which sometimes lead to death of the patients from exhaustion or as Griesinger and Sticker claimed to renewed collapse. It was held that the injudicious use of purgatives, dietary indiscretions or exposure of the patients to cold temperatures or to over-exertion were apt to lead to this kind of relapse As Griesinger maintained, such relapses were sometimes responsible for chronic gastro-intestinal disturbances

As mentioned in the fourth chapter Sticker also referred to the recurrence of cholera attacks in individuals who had recovered from the disease but some weeks previously (*Spätrecidive*) He admitted that such early recurrences of cholera were not related to the initial attacks but were the result of reinfections. It is noteworthy that such reappearances of the disease, which Sticker himself conceded to be rare were mostly recorded before the discovery of *V cholerae* offered possibilities for an exact diagnosis of cholera.

Complications

General considerations

Etiologically the complications met with in individuals who are recovering or who have recovered from cholera fall into two categories (*a*) complications spontaneously arising either as immediate sequelae of the choleraic process or owing to the presence of secondary infections or other concomitant morbid processes and (*b*) complications whose cause lies in inadequacies of the infusion treatment (to be dealt with in a later part of this chapter)

Macleod (1910) made an interesting but (in the light of further information) not invariably adequate attempt to classify the complications spontaneously arising into the following three groups

(1) Complications of a *functional* character comprising conditions such as anaemia, debility nervous depression, jaundice, gastric irritability persistent hiccough, insomnia dementia, paraplegia, anasarca, irregularity of bowels, chronic diarrhoea, and abortion (or miscarriage) in pregnant women.

(2) Complications of an *inflammatory* nature, including oedema of the lungs (?) bronchitis pneumonia pleurisy meningitis, conjunctivitis, arthritis, parotitis and dysentery [1]

(3) *Destructive* processes, such as ulcerations of the cornea bed-sores, gangrene of the nose, ears, penis and scrotum or more rarely of the fingers and toes.

It is consoling to note that nowadays, owing to prompt and adequate treatment and proper general management of cholera patients, several of the complications enumerated by Macleod or other early writers have ordinarily become exceptional or are even no longer encountered at all This is particularly true of "destructive" processes such as gangrene and bed-sores That, however if it cannot be checked, cholera may still lead to such ravages is exemplified by observations made by Wardener (1946) in a group of prisoners of war who lived, and whose ailments had to be treated, under most distressing conditions As he recorded

> "Large, painless, black and rapidly growing areas appeared at all pressure points mainly across the sacrum and upper back. In 24 hours an area might be formed 3-4 [inches (7.5-10 cm)] in diameter After two or three days the gangrenous areas would begin to separate. In a few cases the whole patch would slough, but the patient always died before epithelisation. The pain attending the separation of the slough, the huge raw weeping areas which followed, the inadequacy of dressings, of nursing and comfort, all combined rapidly to exhaust the patient. In others there was no reaction the gangrenous area remained, becoming more extensive until death."

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[1] Macleod statement that dropsy has been described as a result of consecutive nephritis can no longer be accepted, because in the unanimous opinion of modern observers chronic nephritic processes never develop as the result of cholera

Such skin gangrene which was always a fatal sign was most marked in sufferers who had been debilitated by the pre-existence of chronic dysentery or beriberi, but was by no means met with in them alone.

In some of Wardener's patients multiple skin abscesses, showing little reaction, developed. In one of these sufferers an abscess on the back became so enormous that one pint of pus was voided after incision

Diphtheric processes on the mucous membranes of the mouth, fauces and the superficial parts of the female genital apparatus due to secondary infections, also seem to have become rare in the cholera patients seen nowadays. Whether one such patient, stated by El Ramli (1948) to have an "ulcer of the tongue" had such a diphtheric lesion is not certain

Parotitis leading to abscess formation evidently quite frequent in past cholera outbreaks, is also of rare occurrence according to recent observations Thus, Tao and colleagues (1948) noted this complication but once in their series of 687 cholera patients, while El Ramli encountered it twice in 689 sufferers (once in the form of a bilateral affection)

The various complications classified as functional by Macleod seem also to have become rare recently under usual conditions. Still, some instances of mental derangement are mentioned in recent publications. Thus El Ramli twice recorded the presence of post-choleraic psychoses while Baligh (1948) referred to three cholera patients in whom a maniacal condition developed and in two of whom death ensued This writer also stated that he had seen signs of a temporary paraplegia in one of the cholera patients under his observation.

It is of considerable interest to note that Huang & Mao (1947) observed 11 cholera patients who immediately after recovery developed a complete flaccid paralysis of the neck and extremities associated with loss of the tendon reflexes, as well as with a slow and weak action of the heart, which responded well to treatment with potassium. An identical syndrome had been previously described under the name of "Pa Pin" (transient paralysis) in individuals not suffering from cholera and had been ascribed to the consumption of barium-contaminated table-salt, the barium supposedly disturbing the potassium metabolism The two authors considered it probable that in the case of the cholera patients exhibiting signs of "Pa Pin" (who had been kept on a salt free diet and had been treated with saline solutions containing no barium) dehydration and acidosis played a causative role in the production of the transient paralysis by (*a*) mobilizing the barium deposits in the bones and (*b*) causing a loss of potassium from the tissues

The following complications, which either remain more or less frequent or are of special interest, deserve separate consideration

Digestive system

As will be gathered from Macleod's summary and from statements made earlier in this chapter various gastro-intestinal disturbances parti

cularly diarrhoea gastric irritability or anorexia may persist during and sometimes well beyond the period of convalescence from cholera but it would seem that apart from a more or less pronounced looseness of the bowels less mention is made of them now than in the past However as shown by the experiences of Wardener, such manifestations are apt to be conspicuous in individuals contracting cholera while suffering from general nutritional disturbances such as beriberi or from malaria or gastro-intestinal infections especially amoebic or bacillary dysentery which *per se*, have a great tendency to become chronic He frequently observed under these circumstances the persistence of diarrhoea and also referred to the occurrence of tenacious anorexia, for which however the not only inadequate but repulsive fare offered the cholera convalescents was co-responsible

Since, as has been discussed in Chapter 6 macroscopically manifest signs of cholecystitis were comparatively seldom met with at autopsy of cholera victims, it is not surprising to find a similar infrequency of clinical appearances pointing to gall bladder inflammation Still observations on this point have been recorded by some authors Reference has been made already in this connexion (see Chapter 6) to Valk s (1915) record on a cholera convalescent who had to be operated on on account of cholecystitis due probably to an invasion of the gall bladder by *V cholerae* Rogers (1921) after referring to the occasional observations of a macroscopically manifest cholecystitis at autopsy of cholera victims stated that

" More rarely this lesion may be sufficiently extensive to produce clinical symptoms of cholecystitis, and I have several times been able to diagnose the condition during life, in the later stages of cholera, in the form of an enlarged and tender gall-bladder palpable in the right hypochondrium. It nearly always subsides within a few days under fomentations, although I know of a private patient of a surgeon, who was operated on by him for cholecystitis during convalescence from cholera "

Making a similar statement in 1952, Rogers estimated that clinically recognizable features of cholecystitis were met with in about 1% of the cholera patients.

El Ramli (1948) seems to have noted clinical evidence of cholecystitis in five (i.e., in about 0 7%) of his 689 cholera patients and to have found enlargement of the liver in two others.

Though it is generally agreed that appearance of jaundice is a rare complication of cholera, its presence has been recorded upon several occasions. Sticker (1912) stated in this connexion that

" Icterus is an extremely rare complication in cholera attacks occasionally it seems to be more frequent. Montefusco [1888], who observed during August and September 1887 at Naples among a little more than one hundred patients five such cases, was struck by this frequency and considered it accidental, since he saw in 1884 among a far larger number of cholera cases only one with icterus. In all five cases the liver became enlarged, the urine had a brown colour and contained bile pigment. The icterus lasted 5 days. Galliard [1892c] found at Paris in 1892 among 380 cholera cases 6 with icterus, four times as a simple symptom of the attack, and twice in the reaction stage as a sign

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monia four times only and of bronchitis twice in a series of 689 such sufferers. Identically Tao & co-workers (1948) found in Shanghai only two instances of pneumonia (one of the lobar and the other of the lobular type) among their 687 cholera patients

Further referring to the problem under review, Rogers (1921) maintained that in cholera patients

"Pneumonia in particular tends to run a very rapid course, an increase in the rapidity of the respirations leading to the detection of a patch of consolidation one day in a patient who appeared to be almost convalescent, while on the following day it may prove fatal. Recovery however not infrequently takes place, although it is always a serious complication."

Rogers (1952) felt entitled to assert that the seriousness of lung complications in cholera was

"associated with much toxaemia due to the presence of large numbers of comma bacilli in the lesions. These cases commonly terminate fatally within a day or two of the appearance of the lung trouble and this complication accounts for a mortality of 3-4 per cent in severe hospital cases."

To consider this statement generally valid is impossible It is true that as has been discussed in the sixth chapter an invasion of the lung of cholera victims by *V cholerae* has occasionally been recorded Since, however a vibrionaemia, which may lead to such a localization of the causative organisms, is not by any means of common occurrence, but, on the contrary exceptional it cannot account for the frequent occurrence of lung complications in cholera. There is consequently not the least reason to doubt that these complications are mostly the result of *secondary* infections

It is of great historical interest that an analogous contention had already been made by Griesinger (1857) Refuting the postulation of some writers that, by analogy with pneumotyphoid, a kind of "lung cholera" existed, Griesinger declared that

"One must object to the idea of such an immediate localization and must rather assume that all these lung alterations are of a quite secondary kind should such a primary lung cholera exist, pulmonary and bronchial affections could not be so much rarer in many epidemics, whereas the great preponderance of gastric affections shows a quite specific trend towards the intestinal mucosa." [Trans.]

Indeed, as Griesinger postulated, the unequal frequency of pulmonary affections in different outbreaks or one should rather say in different climates, militates against the assumption of their direct causation by *V cholerae*

As will be discussed in a later part of this chapter the development of lung oedema in the reaction stage of cholera is unfortunately quite often connected with the injudicious administration of infusion fluids However clinical observations as well as post mortem findings made in the past leave no room for doubt that the presence of pulmonary oedema is by no means

of a general infection with fever profound prostration and generalized erythema there was no bile pigment in the urine. Autopsy in these two cases showed cholecystitis and angiocholitis suppurativa. Hesse [1909], at St. Petersburg during the years 1908 and 1909 found icterus 8 times during the attack or the period of convalescence in 1156 cholera patients.

"Icterus is more frequently seen in the reaction stage or during convalescence than during the attacks." [Trans]

The occasional presence of jaundice was also referred to by some subsequent cholera workers, for instance by Piras (1913) who made the unique claim that all convalescents continuing to show *V cholerae* in their stools for prolonged periods had transient icterus. El Ramli (1948) noted the presence of icterus in but one of his numerous cholera patients.

Circulatory system

As discussed above, cardiac alterations may lead to sudden death of cholera convalescents from heart failure. Another interesting, but apparently quite rare sign of cardiac impairment in cholera convalescents is bradycardia, which in the experience of Wilkinson (1943) sometimes became rather marked (40-50 pulse beats per minute) As noted above, Huang & Mao (1947) also referred to the presence of this sign.

Respiratory system

Though the occurrence of various complications in the respiratory system of cholera sufferers has been described, only bronchitis, the two types of pneumonia and, as will be discussed later lung oedema are regularly met with, while others, like pleurisy empyema, lung abscesses and infarctions, are rare Even the statements regarding the frequency of bronchitis and pneumonia vary considerably no doubt because their incidence is governed by climatic factors Reference has been made already in this respect to the frequency with which Simmonds (1892) and again Stoerk (1916) both working in Europe found pneumonic foci at autopsy of cholera victims. Similarly Rogers (1921) drew attention to a statement of Wall (1893) according to which the incidence rate of pneumonic complications during the 1892 Hamburg epidemic was no less than 41%, whereas such lung processes were far less common in the cholera patients of India. Referring to his personal experiences, Rogers added that

"In Calcutta I have lost only from 3 to 5 per cent of my cases from these [i.e., pulmonary] affections, while they have been less frequent since a more airy ward has been available for the treatment of cholera patients."

As a corollary to these findings Arzt (1914) working during the First World War at Cracow (now in Poland) was impressed by the frequency of pulmonary complications, including bronchopneumonia, in his cholera patients, whereas El Ramli (1948) in Egypt noted the occurrence of pneu

Ear inflammations only occasionally observed in the past (particularly in connexion with faucial diphtheritic affections or purulent parotitis) have nowadays become even more rare complications of cholera Thus El Ramli recorded but one instance of otitis media in his series of 689 patients

The difficulties of hearing noted in cholera patients by Wardener (see page 705) and also by a few earlier observers invariably disappear with the progress of convalescence.

Urinary apparatus

Complications developing independently in the urinary apparatus of cholera patients are quite infrequent. Indeed, a convincing corollary to the now generally accepted belief in an extrarenal origin of the post choleraic uraemia is that cholera attacks lead only with extreme rarity or—as almost all modern workers maintain—even never to nephritic alterations of a chronic nature Sticker stated in this connexion that

> "Chronic morbus Brighti with hydrops as a secondary disease of the cholera attack has only been observed quite exceptionally the more frequent is long lasting oedema of the legs without nephritis as a sign of cholera marasmus." [Trans.] [1]

Other rare complications noted in the urinary apparatus of cholera convalescents comprised cystitis, kidney abscesses due to an ascending infection (one observation by Rumpel 1894) and pyonephrosis, once recorded by Wilkinson (1943)

Sticker maintained that disturbances of the function of the urinary bladder were not rare after cholera attacks. These consisted of

> "pains in the urinary bladder due to exsiccation of the mucosa or the violent contraction of the muscularis further sphincter paralysis with incontinence of the urine bladder paralysis with urinary retention or ischuria paradoxa." [Trans.]

Usually however such functional disturbances were but temporarily manifest so that, as Sticker put it, their prognosis was not bad.

Genital organs

Apart from gangrene of the penis or scrotum, observed in the past but hardly ever nowadays, no complications have been met with in the genital organs of male cholera patients. However the complications apt to develop in the female genital organs are of great and often even of ominous importance

In agreement with observations on this point made in cholera victims (see Chapter 6) Griesinger stated that

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[1] Sticker belief in the non-renal origin of the leg oedema becoming manifest in cholera convalescents has been shared by subsequent authors, e.g., by Takano and colleagues (1926). Dunlop (1946), who observed development of oedema in almost all the cholera-affected prisoners of war under his care, ascribed it to protein deficiency

invariably the result of such treatment In agreement with these records, El Ramli (1948) found lung oedema to be present in three cholera victims to whom no treatment had been given Only two of his 13 cholera patients in whom clinical evidence of pulmonary oedema had been found recovered the other 11 died between the third and 18th day of illness, at an average after 11 days. This high mortality noted also by other observers, is not surprising in view of the fact that, as recently confirmed by Chakravarti & Chaudhuri (1954) pulmonary oedema often becomes manifest in the course of post-choleraic uraemia.

Meningitis and meningismus

While meningitis, mostly the result of secondary infections appears to be a rare complication of cholera, signs of meningismus are quite frequently met with in children affected with this disease. Sticker (1912) referred in this connexion to observations of Niemeyer (1832), according to whom in such children the development of hydrocephalus acutus led to rapid death in spite of an otherwise favourable reaction However in the experience of other workers also quoted by Sticker this condition though leading to profound sopor was not necessarily fatal as long as the secretion of urine remained satisfactory

Sensory organs

Referring to eye-complications in cholera, Sticker (1912) stated that

"One observes not rarely in the eyes of cholera convalescents a keratitis, which commences in slowly progressing attacks as a consequence of corneal exsiccation and progresses during the following days. It is first manifested by the appearance of small whitish areas of turbidity which, accompanied by slight injection of the adjacent parts of the conjunctiva, form mainly in the lower half of the cornea, because this has not been covered by the eyelid. Within a few days these areas of cloudiness become confluent and form a white spot in front of the pupil, which diminishes or abolishes vision. If the process is not interrupted by a fatal issue, small ulcers form, which may subsequently exhibit all manifestations and sequelae of an ulcus serpens." [Trans.]

Sticker added that a persistence of the amaurosis, which sometimes suddenly developed during acute cholera attacks, was rare because most of the patients thus affected succumbed before they had reached the reaction stage.

The development of corneal ulcerations, though not as common as in the past, has still been noted by modern observers. For instance El Ramli (1948) recorded having met with this condition (three times unilaterally and twice bilaterally) in five of his patients

Rogers (1921) dealing with the sequelae of cholera attacks stated that

"Sloughing of the cornea occurs in the late stage of severe attacks in old and weakly subjects, who have long lain in a semiconscious state with the eyes half open. It affects the lower segment of the cornea, and, according to Goodeve [1866] it may readily heal in recovering cases without serious disfigurement. It has disappeared since hypertonic transfusions have been in use"

week after admission, while the other four were delivered of living children one during her stay in hospital three after they had been discharged

Referring to earlier observations in France since 1866 Galliard (1892a) stated that according to most of them cholera-affected pregnant women almost invariably died, an overwhelming majority after they had had abortions or miscarriages. In one instance a caesarian section was per formed *post mortem* on a 8½-months pregnant woman who had succumbed to the disease in less than 24 hours, but the foetus was delivered dead. However, in contrast to these unfavourable experiences, Galliard quoted, without furnishing an exact reference, the following statement of Moutard Martin

"I have attended many women struck [by cholera] at all the stages of pregnancy and the number of those who have succumbed after premature birth or abortion has been minimal. In one female, affected 8 days before the term of pregnancy by algid cholera provoking premature delivery the illness rapidly terminated in a favourable manner Among the women delivered before term under the influence of the disease, one only succumbed." [Trans.]

Galliard s personal experiences were far less favourable than those of the observer just quoted. For including two patients mentioned by him in a second paper (1892b) only two out of the *nine* pregnant women under his care survived (mild) cholera attacks, one being discharged well in the sixth month of pregnancy the other having been delivered of a living child while still in hospital. It is remarkable that no less than five out of the seven pregnant patients succumbing to cholera died without having been delivered.

Since the idea of saving the life of cholera affected pregnant women by the induction of labour had been entertained by some writers, it is important to note that in Galliard s opinion this procedure while offering hypothetical chances of survival for the child if resorted to near term, would certainly (*à coup sûr*) kill the mother Hence he said, "I consider that there is only one thing to do not to interfere (*s abstenir*)" The same opinion was also expressed by Kovalevski (1894) and by Schütz (1894) who recorded that 28 out of 50 pregnant women, who out of a total of 115 such patients survived cholera attacks could be discharged undelivered. While Basil (1910) also advised against interference if pregnant women contracted cholera, Lowell (1917) maintained, on the contrary that

"The essential factor in the treatment of pregnant cholera cases is to remove the dead foetus as soon as possible and in the manner best suited to the mother s condition, because it shortens the period of convalescence, preserves the strength of the mother and reduces the mortality to about that of the nonpregnant cases"

It may be conveniently added that according to Galliard (1892b) cholera was by no means particularly fatal in women who contracted the infection while nursing their babies out of 10 such patients only four died, the mortality rate being thus below the general average of 50%.

Like some previous workers, Galliard noted that in several of these patients the cholera attacks did not disturb the physiological function of the

"As early as the attack stage there is often marked congestion and haemorrhage in the internal genitalia, particularly in the ovarian follicles. Similarly blood excretions take place in the reaction period which are not coincident with the menstrual periods and also occur in old women a partly catarrhal and partly diphtheritic process with *haemorrhagic infiltration of the uterine mucosa is frequent in the case of protracted* reactions and may—sometimes with marked intensity—spread so far down into the vagina as to become visible If somewhat more markedly developed, these processes give a very bad prognosis" [Trans.]

Fortunately the more serious alterations described above are nowadays of historical interest rather than of actual importance.

Continuing his masterly description Griesinger recorded that

"Pregnancy enhances the danger of cholera but not invariably to a very considerable extent. Sometimes the disease terminates favourably with subsequent birth of a healthy child, *more frequently it leads to abortion followed by death or recovery of the woman*. Many die without having aborted. The statements of the various observers regarding the frequency of abortion are very discrepant abortion is said to be more likely after the fifth month of pregnancy and to occur the more rapidly the more violent are the muscular cramps If lying-in women are attacked by cholera, generally a very serious often rapidly fatal, illness results the lochiae may cease to flow or may continue the milk secretion ceases but rarely in a complete manner croup of the uterus is frequent in lying-in women or those who have aborted Cholera is almost without exception rapidly fatal in those attacked when suffering from puerperal fever" [Trans.]

Supplementing these statements, Sticker noted that Drasche (1860) had demonstrated the presence of urea in the milk of a lying-in woman He also drew attention to the claim of Marcus (1832) that breast feeding of babies during or after cholera attacks of the mothers or wet nurses did not endanger the infants.

Early statistics compiled by Bouchut (1849) and by Hirsch (1855) respectively and quoted by Griesinger and by Sticker had shown that

(*a*) out of 50 pregnant women contracting cholera 25 had abortions 9 of the latter died as against 19 of the 25 women whose pregnancy had not been interrupted and

(*b*) abortions or miscarriages were observed in 17 out of 25 women contracting cholera when pregnant 11 of the former died, while out of the 8 women not delivered 5 succumbed.[1]

Further valuable information on the subject under review became available during the 1892 cholera outbreaks As noted in the sixth chapter Tipjakoff (1892) observed a fatal issue in six out of seven cholera-affected pregnant women, all of whom had abortions or miscarriages

Klautsch (1892) reported that out of the 10 cholera affected pregnant women he saw five died (one undelivered, four after they had had abortions or miscarriages) Only one out of the five survivors had a stillborn child one

[1] Further early publications on cholera in pregnant women, which have not been accessible to the present writer are quoted in the *Index-Catalogue of the Library of the Surgeon-General's Office United States Army* Washington, D.C., 1882, vol 3, 147 and 1898, 2nd series, vol. 3 605.

the features of erysipelas. Smaller petechial or larger blood extravasates (vibices and suggillations) also occur Less rarely there are eruptions of vesicles corresponding to miliaria, or formation of pustules (impetigo or ecthyma) The exanthemata are found most frequently on the extremities, but they can appear also on other parts of the body Herpes labialis or facialis in the strict sense is extremely rare. Moreover furuncles appear and a tendency to the formation of these may persist long in the period of convalescence In rare cases one meets with extended phlegmonous inflammations, decubitus and gangrenous processes in the peripheral parts of the body (ears and nose, fingers and toes)." [Trans.]

In Liebermeister s opinion the etiology of these various manifestations was not uniform The appearance of some of them particularly of the erythemata, the furuncles and the gangrenous processes, was due to or was facilitated by the previous circulatory disturbances. In the causation of others, especially of the urticarial rashes, toxins or other harmful substances in the blood might have been involved Erysipelatous and phlegmonous processes were the result of secondary infections, particularly streptococcal invasions. However though admitting a possible role of the cholera toxin, Liebermeister maintained that all the skin manifestations described above merely fell into the category of non-specific sequelae " There is " as he put it, " no specific cholera exanthema "

The subject at present under review continued to be dealt with in quite numerous publications, specially noteworthy among which—besides some already quoted in this chapter—are those of Simond & Pasteur Vallery Radot (1914) [1] Valk (1915) Soucek (1916) Ichikawa (1916) and Dong Noc Dieu & Millous (1923)

One noteworthy fact which a study of this newer literature discloses is that the more serious skin affections appear to have become far less frequent than was the case in the past—no doubt on account of the prompt treatment and better general care which can now usually be accorded to cholera patients.

It is generally agreed that the skin exanthemata, which appear at the end of the first or during the second week after the onset of the disease disappear again after a few days It is also universally held that, with rare exceptions, the appearance of such skin rashes is a prognostically favourable sign Dealing with the evidence available in this respect, Griesinger (1857) considered it an open question

"whether the eruption itself exerts a modifying influence on the intrinsic processes, or whether these must already have taken a more favourable turn by the time an exanthema becomes manifest" [Trans.]

Liebermeister (1896) and, quite categorically Sticker (1912) advocated the latter view which is now generally accepted

Statements regarding the frequency of the skin exanthemata are at variance Griesinger maintained that they appeared more frequently in

[1] See summary in the *Tropical Diseases Bulletin* (1914) 4 324

mammary glands. Similarly Schütz (1894) found that regardless of the severity of the infection milk secretion continued in about half of the nine cholera affected lying in women observed by him during the 1892 Hamburg outbreak.

Turning to recent observations on cholera in pregnant women reference has to be made first to the publication of Baligh (1948) who as quoted in the *Tropical Diseases Bulletin*, found that

"*Abortion and premature labour* were constant in those pregnant women who had cholera. Of 7 such, 4 aborted, 2 had premature labour and 1 died during labour at term All of them died within 2 or 3 days, except 1 who died of pneumonia in 5 days. These abortions were complete and without haemorrhage and were attributed to severe purgation, severe reflex uterine contractions and toxaemia."

Baligh was, like Galliard long before him, averse to any interference when dealing with such patients, the more so because in his experience abortion was always quickly completed

In agreement with Baligh s findings, El Ramli (1948) recorded that out of 14 women admitted with cholera when 2-8 months pregnant, no less than 11 had abortions or miscarriages while in hospital one died there undelivered, and only two were discharged while still pregnant. Abortions or miscarriages took place from the fourth to the seventh day after the onset of illness. It is noteworthy however that a fatal issue was observed in only four of the 11 patients in whom pregnancy had become interrupted.

In contrast to these recent experiences in Egypt Tao and co-workers (1948) recorded only six instances of abortion in 20 cholera affected pregnant women. Thus Griesinger was certainly right when insisting that the frequency with which cholera attacks led to abortions or miscarriage was apt to vary to a considerable degree As far as the present writer can judge it is impossible to adduce valid intrinsic reasons for the occurrence of these marked differences One cannot fail to note in this connexion that the figures on the incidence of abortions or miscarriages submitted by the various workers are too small to have statistical significance.

Skin manifestations

Classical descriptions of the manifestations on the skin of cholera patients or convalescents have been given by Griesinger (1857) and again by Liebermeister (1896) As the latter author stated

"No exanthemata are present at the height of the disease they appear first in the stage of involution [*Rückbildung*] and even then only in a minority of the patients. Frequently blue-red spots become manifest during the reaction on the skin corresponding to areas in which the circulation has not become restored to a sufficient degree. Next in frequency one observes smaller or larger red spots, designated respectively as roseolae and erythemata, and the latter can show various forms, appearing, for instance, as erythema annulare figuratum or multiforme Sometimes nodules are simultaneously present, so that a roseola papulosa is formed or weals are formed so that the appearances of urticaria are present more extensive reddening and swelling of the skin can show

it is not surprising to find that infections with *V cholerae* have often been observed in and have exacted a heavy toll among, patients suffering from pre-existent acute or chronic diseases Among the former Griesinger enumerated typhoid pneumonia, "catarrh of the lungs" (i e., incipient lung tuberculosis) erysipelas puerperal infections, dysentery, relapsing fever, acute rheumatic fever and smallpox, and among the latter chronic gastro-intestinal affections (including gastric ulcer) dropsy due to various causes, tuberculosis, nervous and mental diseases, carcinoma, syphilis and emphysema. Continuing with an impressive account of the influence exerted by subsequent cholera infection upon some of these pre-existing diseases, he stated

"In the case of the acute diseases one sometimes finds a mixture of the progressing phenomena of the old and the new disease and a fluctuation between the two still more frequently the former come to a standstill with the onset of cholera After the termination of cholera they mostly recur complete their course, if acute, or continue for an indefinite time when chronic. In the case of smallpox, pneumonia and similar affections, with the onset of cholera the fever soon ceases, the local process in the lungs does not spread, the exudate remains stationary and is said to be particularly dry after death signs of acute rheumatism often disappear altogether but reappear afterwards the spleen tumour in typhoid is said to decrease rapidly (Hamernik [1850]) In patients with relapsing fever cholera sometimes appears during the attack and then proves rapidly fatal (Heidenhain [1854]) if it begins during apyrexia, it can still come to a paroxysm of fever Extensive pleuritic exudates, marked ascites and general hydrops mostly decrease rapidly with the appearance of purging, but ovarian cysts remain filled of course the hydrops resulting from liver and heart affections and the like afterwards becomes manifest again. A sudden cessation of the sugar secretion in diabetes and its return after recovery were likewise observed Generally speaking, the inspissated blood attracts all the water otherwise used for pathological processes. One likewise observed, together with the onset of cholera, a cessation of pertussis and its later reappearance the disturbances of tuberculosis become minimal, to return afterwards with renewed violence (Dittel [1850]) mental diseases are but rarely influenced." [Trans.]

The problem of the relation of cholera attacks to other acute infections, either pre-existing or appearing subsequently especially dysentery and typhoid, calls for further discussion

The statements made by earlier observers regarding the co-existence of cholera and dysentery have to be interpreted with caution, because patients affected only with the former disease may show clinical features resembling those of the latter Girode (1892) noted in this connexion that cholera vibrios were sometimes detected in viscous stools intermixed with pure blood and that after the death of such sufferers features suggestive of dysentery were met with in the large intestines mainly affected. Similar observations on a "haemorrhagic" form of cholera have also been recorded by some subsequent workers, e g. by Scicluna (1912) and by Rogers (1921) (see Chapter 8) Sticker (1912) maintained in this respect that in some cholera epidemics

"the disease showed in its clinical appearance and in its course a striking similarity with dysentery being associated with rending abdominal pains, incessant defaecation

female than in male cholera patients and, while more frequent in younger individuals, were rare in children. He also noted that the frequency of the skin manifestations varied markedly in different epidemics or even in different stages of one and the same outbreak While quoting identical experiences, Sticker (1912) pointed out that the statistics furnished in this respect by different observers were not uniformly comparable.

Arzt (1914) who observed exanthemata in three out of 25 cholera patients (in 20 of whom the diagnosis had been bacteriologically confirmed), considered the incidence of these affections to be high an opinion also expressed by Soucek (1916) Ichikawa (1916) claimed that skin rashes, appearing in cholera patients who had passed the critical stage of the disease showed a total incidence of 78%, but became marked in only 5%. Studying 207 seriously affected cholera patients (only 58 of whom recovered) Murayama (1917) found skin rashes in 6 7% of all the sufferers

A definitely low incidence of the exanthemata was noted by Valk (1915—eight instances of urticarial rashes in about 115 cholera patients) by Dong Noc Dieu & Millous (1923—seven positive observations in 129 patients) and by El Ramli (1948) who recorded the presence of various skin rashes, apparently including herpes, in only six of his 689 patients.

The question of the etiology of the skin exanthemata is still under debate. Some of the modern observers, e.g., Arzt (1914) ascribed their appearance to an action of the cholera toxin others, like Ichikawa (1916) and Soucek (1916) considered them anaphylactic phenomena. However while it would be unwarranted categorically to rule out these possibilities, which had already been envisaged by Liebermeister (1896) it is most likely that an important, perhaps even a preponderant role in the production of these skin manifestations is played by the administration of inadequately prepared saline solutions. Strongly in favour of this postulation are the facts that (*a*) Valk (1915) noted an appearance of urticarial rashes not only in some of his cholera patients but also in other individuals whom he had treated with hypertonic saline but in whom the presence of *V cholerae* could not be demonstrated even after repeated examination and (*b*) Tao and co-workers (1948) who were aware of the reactions produced by pyrogen-containing saline solutions and avoided their use later in their work, evidently never observed the appearance of skin rashes in their numerous cholera patients. It is likely that at least some of the skin rashes observed by early workers, who did not resort to saline treatment, were due to the internal administration of drugs such as calomel.

Co-existing diseases

Since, as Sticker (1912) aptly stated, cholera

"attacks with predilection sick and weakened individuals and makes, as it were, a selection of the invalidated members [*Anbrüchigen*] of the population" [Trans.]

In the experience of Rogers (1921 1952) the life of cholera patients contracting dysentery in the convalescent stage was not endangered, because such complicating infections yielded readily to simple treatment As has been noted above (see page 719), Wardener (1946) found on the contrary that the appearance of cholera in patients who had been weakened by pre-existing amoebic or bacillary dysentery was fraught with serious consequences. Similarly, Chatterjee et al (1955) stated that in a series of cholera patients one "of the most important causes of the severity of symptoms" was the co-existence of amoebic dysentery infection They added that many of the patients in question showed a peculiar blue coloration of the tongue

Important observations on the co-existence of cholera and typhoid infections may thus be summarized

Author	Findings
Lebert (1874)	Found that typhoid patients, if subsequently attacked by cholera, almost invariably died
Girode (1893)	Stressed that both clinical and post mortem signs suggestive of typhoid had been found by him in several individuals infected only with *V cholerae* Consequently one was entitled to speak of a mixed infection only if in addition to this organism, *E. typhosa* could be isolated either during the life of the patients or from autopsy materials. Referred to two instances in which such a true mixed infection had been present. One of these sufferers, personally observed by Girode, had apparently contracted both infections simultaneously signs of typhoid becoming manifest after the acute cholera attack had subsided. Death occurred after an illness of 10 days typical gross signs of typhoid were found at autopsy and *E. typhosa* was isolated in pure culture from the swollen spleen.
Doerr & Weinfurter (1914)	Gave a detailed account of a patient who was admitted with typical signs of typhoid, but at the end of the second week of illness suddenly showed signs of collapse with a rapid drop of the temperature and rice-watery stools and died on the same day In the intestinal contents obtained at autopsy both cholera vibrios and typhoid bacilli were found the latter were also isolated from the bile and spleen. It could not be decided whether this individual was a cholera carrier who had afterwards contracted typhoid infection or as was more probable, he became infected with *V cholerae* while in the incubation stage of typhoid fever
Jacobitz (1915)	Referred to an instance of mixed typhoid and cholera infection which was identical with that just described. It is interesting that the rice-watery stools of both these patients showed a yellowish tint.

and tenesmus, frequent haemorrhagic-purulent dejecta, and lasted for a week or longer very slow recovery taking place instead of the development of a reaction." [Trans.]

It is, however rather doubtful whether such patients suffered from cholera alone Sticker himself conceded the difficulty of differentiating clinically the above syndrome from dysentery and admitted that the latter infection might have been co-existent with cholera in some of the out breaks mentioned above. It is certain that such an association of cholera and dysentery has been repeatedly observed, particularly often during the First World War at autopsy by Stoerk (1916) and clinically by several other Austrian or German workers (see summary by Meggendorfer, 1918) The following of these observations, which have invariably been confirmed by laboratory examinations, deserve mention

Author	*Findings*
Bujwid & Arzt (1914)	Stated, referring briefly to patients who had previously been infected with bacillary dysentery (Shiga-Kruse type) and had afterwards contracted cholera, that the prognosis of such mixed affections was not necessarily unfavourable. For instance, out of three such sufferers only one died, while the other two recovered.
Aronson (1915)	Referred, besides briefly mentioning one instance of mixed dysentery and cholera infection, to another patient who showed clinical features of cholera but from whose stools only dysentery bacilli could be isolated. (Considering, however the occasionally great or even insurmountable difficulties of arriving at a laboratory diagnosis of cholera, the absence of a mixed infection should not be rashly asserted.)
Walko (1915)	Commented upon six instances of mixed dysentery and cholera infection by stating "Besides the mostly grave cholera symptoms, signs of dysentery were manifest as well, the most marked of which were blood-containing stools. The patients suffered to an extraordinary degree from the double infection and it was amazing that, in spite of a high degree of debility their life was spared. Recovery was very slow" [Trans.]
Baerthlein & Grünbaum (1916)	Found evidence of mixed dysentery and cholera infection in 15 patients, only one of whom succumbed, and stated that "A minority of these mixed infections were merely bacteriological complications, i.e., the complicating infection—dysentery—was manifested solely by simple excretion of dysentery bacilli in the majority specific, clinical features were observed, e.g., in dysentery admixture of blood to the stools " [Trans.]. Sometimes the cholera patients showed passing fever when becoming additionally affected with dysentery

Note Reference will be made below to some further observations on this point by Meggendorfer (1918)

by Meggendorfer (see above) and previously by Mancini (1913) and by Jastrowitz (1916) The records of the two last mentioned workers may be summarized thus

Mancini (1913) dealt with a 14-year-old girl admitted seven days after she had fallen ill with moderately high fever some meteorism, spleen tumour and pea-soup-like diarrhoeic stools. Positive agglutination tests established the presence of a paratyphoid B infection. Nine days after admission signs of collapse became manifest, the temperature in the axilla dropping to 35.2 C. Diarrhoea became more frequent, the stools became rice-watery and were found to contain cholera vibrios. Two days later the patient died. In Mancini s opinion she had been a carrier of *V cholerae* subsequent paratyphoid infection leading to a manifestation of clinical signs of cholera infection.

As described by Jastrowitz (1916), a man who fell ill with signs of bacteriologically confirmed cholera showed a temperature rise and an incipient spleen tumour three days after onset. At the same time the stools became faeculent, but still contained cholera vibrios. After the fever had continued for about a week, paratyphoid B bacilli could be cultivated from the blood of the patient and at the same time *V cholerae* was isolated from the stools for the last time. About a week later the fever ceased, the stools became solid, and the patient felt well, though weak. He had, however two relapses, during which the stools, though containing neither cholera vibrios nor paratyphoid bacilli, were diarrhoeic and contained small mucus particles and blood. On both occasions paratyphoid bacilli were found in the urine. Though no further relapses occurred, the convalescence of this patient was still interrupted by the appearance of febrile bronchitis and final recovery began only about two months after the onset of the initial cholera attack.

In the opinion of Jastrowitz the cholera and the paratyphoid infections of this patient had taken place at one and the same time.

The simultaneous occurrence of cholera and relapsing fever was recorded during the First World War by Russ (1915) and by Walko (1915) The latter observed 16 patients admitted with cholera in whom relapsing fever attacks appeared afterwards as well as four sufferers in whom both infections were already manifest at the time of hospitalization A few instances of triple infection with cholera, typhoid and relapsing fever were also observed

Unusual forms of cholera

Cholera siderans (cholera sicca)

Some of the early observers of cholera outbreaks both in India and in Europe drew attention to the occurrence of a rapidly fatal type of the disease to which since purging and vomiting appeared to be absent, they gave the name of cholera sicca in preference to the less frequently used but—as will be shown below—more adequate designation of cholera siderans.

Dealing with this form of the disease at a later date Griesinger (1857) made the following statement

" As to cholera cases without any evacuations, the *cholera sicca*, one cannot totally deny their occurrence however as has been established at Moscow in 1830 (Jähnichen

Author	Findings
Walko (1915)	Observed a considerable number of patients in whom cholera infection became manifest at the acme or during the decline of typhoid fever If a severe cholera attack resulted, the signs of typhoid fever were altogether replaced by those of cholera gravis, but it is noteworthy that several times the onset of the latter disease was associated with profuse intestinal haemorrhages. The combination of typhoid with severe cholera was not invariably fatal the patients who survived no longer showed marked signs of typhoid fever but relapses of the latter affection were sometimes observed. Cholera attacks of moderate severity exerted a less profound influence on the pre-existing typhoid affection, the signs of which once more became marked as soon as the reaction stage of cholera had been reached. Slight cholera attacks or the development of a cholera carrier state did not modify the course of the pre-existing typhoid affections. Secondary infections of cholera patients with typhoid were much more rare than those described above. However cholera carriers more frequently contracted infections with *E. typhosa*.
Baerthlein & Grünbaum (1916)	Referred to eight patients showing evidence of mixed cholera and typhoid infection (one fatality) as well as to two sufferers in whom the first-mentioned infection was associated not only with typhoid, but also with dysentery one of these sufferers with a triple infection recovered, the other died after an illness lasting two weeks. The majority of the cholera- and typhoid-infected patients showed either slight fever for several days or serious signs of typhoid, so that, as the authors put it, the cholera affection was relegated to the rank of a subordinate complication. It is noteworthy that cholera vibrios were invariably detected in the stools before typhoid or dysentery bacilli could be cultivated.
Meggendorfer (1918)	Observed in a group of soldiers, who had consumed lettuce cleaned with contaminated water 20 pure cholera infections and 5 typhoid or paratyphoid infections, and the following instances of mixed infections cholera+typhoid or paratyphoid (14 times), cholera+dysentery (once) and cholera+paratyphoid+dysentery (3 times). Out of these 18 patients showing evidence of mixed infections, none died 12 had severe and 5 slight cholera attacks, while one was merely a carrier of *V cholerae*

Note A further observation by Ling (1932) has already been referred to in the sixth chapter

In contrast to the ample experiences recorded above, observations on mixed cholera and paratyphoid infections seem to have been made only

A unique instance of recovery of a patient showing typical features of cholera siderans (absence of purging, but collapse and presence of fluid in the intestines) was recorded by Craster (1913)

The assumption of Griesinger and some subsequent authors that in the type of cholera at present under review an ileus-like intestinal paralysis accounts for the absence of purging appears to be adequate Since however in the patients attacked in this manner a transudation of fluid into the small intestines invariably occurs, they cannot be said to suffer from a "dry" form of cholera The designation of cholera sicca should, therefore be given up in favour of that of cholera siderans, which, while not conveying an erroneous impression regarding the underlying morbid process, appropriately draws attention to the almost invariably rapid death of the sufferers affected by this type of the disease

Cholera in children and aged persons

Though some reference has already been made in the foregoing pages to cholera in children and aged people, and although this subject will have to be discussed further when dealing with the problems of epidemiology in the following chapter it seems necessary to describe at the present juncture the clinical peculiarities of the disease in these two age-groups in a comprehensive manner

Children Dealing with infantile cholera, Griesinger (1857) stated that

"In newborn and very young children in general, the course is often rapid, showing rather the signs of a very quick exhaustion through the evacuations than the characteristic symptoms of cholera there is often no prodromal diarrhoea vomiting is also not a constant feature of the attacks. Watery purging is invariably present, the children become very restless, the face becomes wan and bluish, the skin and musculature are withered, cramps are rare, cyanosis is not marked. The children become soporous and cold and lie prostrate with weak respiration and heart activity open and staring eyes, toneless voice and no urine. Sometimes a typhoid state or a stage of exhaustion resembling the hydrocephaloid with sopor and slight convulsions, but with satisfactory urine secretion, subsequently develop which are often followed by recovery" [Trans.]

Generally speaking, however the prognosis of cholera in children was unfavourable over 90% of the newborn and about 70% 80% of children under 4 years not surviving the attacks according to Griesinger the mortality of older children up to 10 years was also above the average

Making a special study of infantile cholera in the Philippine islands, McLaughlin (1909) reached the conclusion that, owing to its frequently atypical appearances, the presence of the disease in children

is often unrecognized and unreported as such, being reported as acute or chronic enteritis, gastro-enteritis, entero-colitis, dysentery acute or simple meningitis, and probably also as infantile beriberi, convulsions of children, and other diseases"

McLaughlin maintained in particular that, owing to the frequency of cerebral manifestations in cholera affected children they were often sup-

[& Marcus]), examinations of the abdomen and autopsy show that a serous transudation into the intestine has taken place, but has not been voided. One has to consider this as essentially due to a paralytic state of the intestinal musculature (? in some cases to a rectal spasm). Sometimes, however the transudation appears to be more scanty than usual or merely of a mucoid character (Parkes [1847]) Cases with such a course, in which the appearances of a cholera attack develop without evacuations, invariably end un favourably they occur particularly in old, debilitated individuals, in whom a slight amount of intestinal transudation suffices to cause the profoundest exhaustion." [Trans.]

Again discussing the problem of the so-called dry form of cholera, Rogers (1921) stressed that in the experience of most Anglo-Indian obser vers, e.g., of Annesley (1829) and Macpherson (1866) this type of the disease was extremely rare. Moreover he drew attention to a statement of Goodeve (1866) according to which even though purging could be absent in rapidly succumbing cholera patients,

"It does not follow that because there has been no purging there was no exudation into the intestines. The exudation is sometimes poured out and retained there, and we should never for a moment confound exudation with purging. In numerous instances in which there has been an absence of evacuations, post-mortem examination has shown the intestines full of fluid."

Rogers drew particular attention to a cholera patient who suddenly died without purging, but whose small intestines were found to be filled with fluid, even though solid faecal matter was present in the large bowel Reiterating a statement he had made in an earlier publication (1909b) Rogers maintained with great reason that

"The very rapid loss of several pints of fluid into the intestines may obviously be more fatal than the gradual evacuation of a much larger quantity through repeated vomiting and purging spread over some days, as in the latter case much loss may be replaced by absorption from the tissues or by fluid given by the mouth and rectum."

Modern observations have confirmed the rarity of cholera siderans. Thus Tao and co-workers (1948) stated that they had not met this type among their 687 patients,[1] and El-Ramli evidently had the same experience Wilkinson (1943) referred to the occurrence of "cholera sicca" in but three of the 547 individuals falling ill with cholera at Hong Kong in 1938. It is noteworthy however that the clinical features in these three patients (two children and one young man) did not fully correspond to those usually met with in cholera siderans For, as Wilkinson described,

"Cyanosis, hyperpyrexia and collapse were the outstanding signs, and one of the children, who had no diarrhoea, showed signs of free fluid in the peritoneum and shrivelled finger tips on admission Her temperature was 106 F All these patients died and vibrios were isolated from the intestinal contents at autopsy Presumably in such cases the outpouring of rice-water fluid into the gut is so rapid that a paralytic ileus ensues, which accounts for a complete absence of diarrhoea."

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[1] However as noted in Chapter 7 one patient obviously succumbing to cholera siderans had been seen in Shanghai by Lieou (1938).

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McLaughlin maintained in particular that, owing to the frequency of cerebral manifestations in cholera-affected children they were often sup-

posed to suffer from acute meningitis whereas—notwithstanding statistics to the contrary—acute meningitis was really a rare disease in Manila. Another source of diagnostic errors was that infection with *V cholerae* could occur in children suffering from enteritis or dysentery without leading to the choleraic syndrome met with in adults. Actually bacteriological examinations of all children supposed to have succumbed to these two diseases or to meningitis during a cholera outbreak showed that the incidence of *V cholerae* infections in children under 10 years of age was 32.5% *being thus considerably higher than the average of 22.8% recorded for this* age group from 1907 to 1909

The observations of most subsequent workers confirmed that the cholera mortality of children under 10 years of age was more or less above the average It is interesting to note in this connexion that Rogers (1921) while in general agreement with this view pointed out that, after improved methods of treatment had been adopted in 1913 the infantile mortality from cholera remained high only in children under 5 years of age (36 4%) whereas the fatality rate in those from 6 to 10 years was lowered to 16 4%, thus comparing favourably with the death rate of 19.2% in the age group from 11 to 40 years. But the fact that 1388 records were available for the latter group as against a total figure of 150 for children under 10 years of age deserves attention.

Recent experiences in China definitely support the view that the death rate from cholera in children was abnormally high. Thus Pollitzer and co-workers (1941) found in a comparatively small but carefully observed group of 324 cholera patients an average fatality rate of 63.2%, whereas that of the 52 children up to 9 years old was 80 8% Tao & colleagues (1948), though in general obtaining excellent therapeutic results (the total death-rate in 687 patients equalling only 4 7 %) were unable to save 14 out of the 78 cholera-affected children up to 10 years old this represents a fatality rate of 17.9 %.

Aged persons General agreement exists that the mortality from cholera is abnormally high in aged persons Griesinger (1857) summarizing the early evidence available in this respect, stated that in this disease the prognosis was best in those from 10 to 20 years old and was still quite good in the following age-group (20-30 years) but then became increasingly less favourable, the fatality rate becoming high in persons over 50 years old and higher still in those over 70 years old, 80%, 90% of whom succumbed. He also drew attention to observations like those of Farr (1852) which showed that aged persons were apt to die from cholera not only more frequently but also more rapidly

Sticker (1912) dealing with the subject under review maintained that

"The behaviour of old and of prematurely debilitated persons in cholera epidemics bears a relation to lessened resistance. If cholera is prevalent, the slightest diarrhoea

in an individual over 60 years is almost invariably a true cholera attack and the slightest attack is extremely dangerous. *Moderate diarrhoeas and slight cramps lead to rapid and* most marked loss of forces and body temperature. If death does not ensue during the attack it is certainly caused through subsequent exhaustion." [Trans]

Similarly Rogers (1921) pointed out that, even after improved methods of treatment had become available the mortality from cholera remained high (49 9%) in persons over 50 years, their low resistance " not giving the treatment the same chance as *in those of more vigorous ages* "

In China, Pollitzer and co-workers (1941) recorded a mortality of 77 8% *in 27 patients of 50-59 years and one of 76.2% in 21 individuals above* the latter age as against an average fatality rate of 63 2% Tao and colleagues (1948) *on the other hand* recorded *only one* death in 20 patients 61 70 years old and none in five individuals of 71 years or more

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

Diagnosis

While it must be fully admitted that it is impossible to arrive at an exact diagnosis of cholera without exact laboratory examinations, it is at the same time of the utmost importance to obtain *prima facie* evidence by clinical methods of the presence of this disease as soon as the sufferers are first seen For as has been discussed in Chapter 7 no reliable rapid method for the recognition of *V cholerae* in their stools is available so that one has to resort to the comparatively tedious procedure of isolating the organisms and identifying them with the aid of serological tests To delay isolation of the patients, immediately followed by appropriate treatment until a final diagnosis has been made in this manner would be fraught with the gravest consequences not only for them but also for their contacts and for the community at large

However while the need for a speedy preliminary diagnosis is obvious, unfortunately it is not uniformly easy to obtain such *prima facie evidence* It is true that, as soon as the epidemic prevalence of cholera has been established, no difficulties will as a rule be encountered in the case of severely affected adult patients The usually sudden onset of the disease with violent purging and, soon vomiting, leading first to the appearance of rice watery evacuations and in due course to the progressive development of marked dehydration, profound collapse and complete, or at least almost complete cessation of the urine secretion, in association with other signs, such as most painful muscular cramps and a characteristic change in the very aspect of the sufferers furnish well nigh unmistakable evidence of the presence of cholera. It is true that as will be discussed below the presence of other

diseases may be manifested by signs more or less resembling those of cholera gravis, but instances in which *all* the classical signs of this disease are met with even though no infection with *V cholerae* has taken place are exceptions rather than the rule

Serious difficulties for a clinical diagnosis may arise however if one is confronted by (*a*) early or sporadic manifestations of cholera, particularly in localities where the occurrence of this disease is unusual (*b*) atypical forms of the disease especially infantile cholera, in which meningeal or cerebral manifestations may overshadow those of the digestive tract or (*c*) mild choleraic affections which, being apt to terminate in recovery without treatment, may be quite easily overlooked, even during epidemics, unless adequate attention is paid to an examination of the stools of the sufferers in question.

As proved by the observations of de Moor (1949) already referred to in the third chapter the clinical features of what he called "paracholera (El Tor)" or more adequately "enteritis choleriformis El Tor" caused by infection with haemolytic vibrios agglutinable with cholera-diagnostic sera, are quite indistinguishalble from those produced by the classical non haemolytic cholera vibrios In agreement with the clinical findings, the "choleriform" outbreaks in the Celebes were characterized by a high fatality rate (75% in 1937 38 and 69% in 1939-40) One must keep in mind, however that, desirable though it is to distinguish between choleriform enteritis and classical cholera through appropriate laboratory tests, particularly haemolysis tests, this differentiation is of no import as far as the treatment of the patients and the control of the outbreaks are concerned.

Considerable diagnostic difficulties may be created during outbreaks caused by the classical *V cholerae* through the occurrence of what has been termed "clinical" cholera, i e., manifestations of the disease characterized by the presence of typical clinical signs of cholera gravis while laboratory tests fail to furnish evidence of *V cholerae* infection. Though the incidence of this "clinical" cholera is not usually considerable, the contrary has been found in some outbreaks. Thus Wilkinson (1943) recorded that in a series of 500 apparently cholera affected patients at Hong Kong positive laboratory findings could be obtained only 349 times. He added that the mortality of the 151 patients showing no laboratory evidence of cholera infection was quite high (47 7 / as against 60 45% in the bacteriologically positive group) Tao and colleagues (1948) stated in this connexion that they could obtain positive cultures from the stools of only 687 patients, while in the case of 934 sufferers showing typical clinical appearances of cholera the diagnosis remained unconfirmed, usually because no laboratory tests had been made, less often because *V cholerae* could not be isolated from the stools upon one or sometimes upon two occasions. Evidently however these workers entertained no doubt that these 934 patients had cholera, and they even claimed that, in view of the epidemic prevalence of the

disease, the same held true of 443 patients without bacteriological confirmation and less clear-cut clinical manifestations of the infection

While one may entertain doubts regarding the validity of this claim there seems to be no reason to deny that most if not all patients seen during outbreaks with typical clinical appearances of cholera but negative laboratory findings have been infected with *V cholerae* the failure to isolate the causative organisms from the stools being due merely to accidental causes. It is noteworthy in this respect that Rumpel (1893), besides recording three instances in which *V cholerae* could not be cultivated from the rice watery stools of patients with clinical signs of cholera gravis, also referred to some other such sufferers whose stools gave alternatively positive and negative, or negative and positive, results. El Ramli (1948) maintained in this connexion that in most of the instances in which a laboratory confirmation of the diagnosis could be obtained

" the cholera vibrios can be isolated from the stools early in the course of the disease. But in other cases they cannot be isolated except at a late period, and sometimes after the stools have become formed "

Evidently El Ramli was able to cultivate *V cholerae* from the stools of 76% of his bacteriologically positive patients within four days of the onset of the disease, in 15% after an illness lasting from five to seven days, and in 9% later once on the 24th day

El-Ramli further referred to 36 cholera victims who had succumbed to typical cholera attacks and at whose autopsy characteristic signs of the disease were found, but from whom no positive cultures had been obtained either during life or after death It is quite likely that in the case of such cholera victims, as well as when dealing with patients showing the signs of " clinical " cholera, a retrospective diagnosis might be established with the aid of appropriate serological tests. However since patients showing clinical signs of cholera gravis must be promptly isolated and adequately treated regardless of whether the presence of *V cholerae* is demonstrated in their stools, such a late confirmation of the diagnosis is of academic rather than practical importance

Differential diagnosis

In order to deal adequately with the various diseases the clinical appearances of which more or less resemble those of cholera, separate consideration will be given to (1) infections produced by vibrios other than the *V cholerae* (2) gastro-enteric affections caused by bacterial species not belonging to the genus *Vibrio* (3) malaria (4) parasitic infestations (5) processes due to the ingestion of certain poisons (6) stokers cramp and allied conditions and (7) a few other diseases

Infections produced by cholera-like vibrios

Though it is impossible to accept all claims made regarding a pathogenetic role of cholera-like vibrios in man, a study of the relevant literature [1] shows that invasions by such organisms may produce manifestations of choleraic disease or as it is often called, of "paracholera" which more or less resemble those of severe cholera attacks. However for various reasons the occurrence of such infections curiously far more frequently observed in areas where true cholera does not regularly occur especially in Africa, is not apt to cause serious difficulties of differential diagnosis. It is true that "paracholeraic" infection sometimes produces syndromes quite closely resembling those of cholera gravis, an evacuation stage—manifested by violent purging with finally rice watery stools, vomiting and muscular cramps—passing into a collapse stage. However even then clinical signs absent or at least rare in true cholera—for instance, colicky pains or fever, or the two together—may almost invariably be observed. In marked contrast to true cholera, even the patients most seriously affected with "paracholera" almost always recover The method of treating such sufferers is identical to that adopted in the case of cholera gravis, and soon after treatment has been initiated the results of laboratory examinations will show the presence of an infection with vibrios other than *V cholerae* However, a laboratory diagnosis of "paracholera" should not be rashly made because (*a*) owing to accidental causes cholera like vibrios may be present in addition to *V cholerae* in the stools of patients suffering from cholera, and may even abound and (*b*) the apparently exclusive presence of cholera like vibrios may merely camouflage infections due to organisms of other bacterial genera, which are apt to remain undetected if attention is focused upon the rapid isolation and identification of vibrios.

Gastro-enteric affections produced by other bacteria

As unanimously stated by numerous observers, gastro-enteric infections produced by organisms other than those of the genus *Vibrio* (particularly often by salmonellae) [2] may produce syndromes more or less closely resembling that of cholera and variously designated as acute gastro-enteritis, cholera nostras, or food-poisoning, and in children often as infantile diarrhoea or as pseudocholera infantum (Sasaki, 1937) As exemplified by Table XX based on an excellent tabulation of Napier (1946), it is usually not difficult to make a differentiation between such affections and true cholera even on clinical grounds However sometimes manifestations

[1] See, in addition to the excellent summary of the early literature by Mackie (1929), [illegible] (1933), Taylor Pandit & Read (1937), Read (1937), [illegible] (1938), Lefrou et al. (1943), Mackie (1946), and Yajnik & Prasad (1954).

[2] Reference to the production of cholera-like syndromes by organisms other than salmonellae has been made more recently by Ghosh (1938—*Ps. pyocyanea* infections) and by Menon (1947—intestinal fuso-spirochaetosis).

TABLE XX DIFFERENTIAL DIAGNOSIS OF CHOLERA FOOD POISONING AND ARSENIC OR ANTIMONY POISONING

	Cholera	*Food poisoning*	Arsenic and antimony poisoning
Epidemiology	Associated with other cases in neighbourhood	Often single group of persons who shared meal no secondary cases	Often one person only
Incubation	*24-72 hours*	*4-24 hours*	*½-2 hours*
Onset	With purging	With vomiting	With burning in throat followed by vomiting
Nausea and retching	None	Yes	Yes, retching marked
Vomiting	Precipitate watery rarely blood continuous	Often single severe vomit mucus, blood streaked	Violent, continuous, mucus, often freely streaked with blood
Evacuation	Early continuous pouring out of pints of watery fluid *inoffensive*	Frequent usually follows vomiting faecal plus blood and mucus, often *offensive*	Delayed single, massive, followed by frequent passing of blood *and mucus*
Tenesmus	None	Yes	Very marked
Abdominal tenderness	None	Marked all over abdomen	Very marked
Dehydration	Very marked	Distinct	Slight
Muscular cramps	Constant and severe	Less constant, extremities only	Severe
Surface temperature	Subnormal	*Often up to* 100°-102°F (37.8°-39°C)	Normal or sub normal
Headache	None	Often	Often
Urine	Suppressed	Seldom suppressed	Sometimes suppressed later
Blood	Leucocytosis mononuclear increase	*Normal*	Slight leucocytosis normal differential

Based on Table VI of Napier (1946), p. 385. An earlier tabulation of the signs differentiating cholera and food poisoning by Tomb (1930) will be found reproduced in the 1931 volume of the *Tropical Diseases Bulletin*

more closely resembling those of the latter disease may be met with. Thus the evacuations, instead of exhibiting appearances fitting Napier's description may be inoffensive and rather similar to those in cholera. It is true that, as has been pointed out by early observers such as Guttmann (1892) they are usually not quite acholic, showing a more or less pronounced yellowish tinge but this is not invariably the case, while, on the other hand, such slightly tinged stools may be voided by cholera patients. Therefore, attempting a clinical differentiation, one should lay no undue stress upon this or other individual signs, but appreciate the condition of the patient in general, paying attention at the same time to the history of his affection. In view of the occurrence of mild or atypical forms of cholera, one should never omit a laboratory examination of the stools of the sufferers, if there is an even remote possibility of the presence of this disease

It deserves special attention that a sudden onset of acute bacillary dysentery may be characterized by signs quite similar to those of cholera. Rogers (1921) stated in this connexion that

> "*Acute Bacillary Dysentery* sometimes presents copious watery stools with marked collapse, and I have known several such cases admitted to the cholera ward. In these cases I have also usually although not invariably found an absence of leucocytosis, while the differential count is quite unlike that of cholera, the large mononuclears not being increased. As soon as a stool has been passed, mucus and also often blood may be detected, and the diagnosis of dysentery can be confirmed by bacteriological examination. It is only very acute bacillary dysentery that I have seen mistaken on first admission for cholera, while if collapse is marked and the blood somewhat concentrated, transfusion is indicated, as in the latter disease."

In agreement with this statement, Strong (1944) quoted evidence to show that in Europe also during the First World War considerable difficulties were encountered in differentiating between the choleraic form of dysentery and cholera. The not infrequent association of both diseases must also be kept in mind to avoid diagnostic errors.

Choleriform malaria

As described by Thayer (1910)

> "The special localisation of grave aestivo-autumnal [malaria] infections in the intestinal tract may result in symptoms simulating Asiatic cholera—sudden, profuse, watery diarrhoea with collapse, the patient sinking before death into an algid condition In other cases, without intestinal manifestations, the paroxysm may be ushered in by a train of symptoms not unlike the algid stage of cholera—*algid pernicious fever*"

A differentiation of the latter atypical form of malaria from cholera ought not to be difficult, both because the patients will not report initial gastro-intestinal disturbances and because the algid stage will be followed in due course by a fever paroxysm. Regarding the first mentioned unusual form of malaria it must be kept in mind that (*a*) in this the typically rice watery stools usually voided by cholera patients are hardly ever met with

(Rogers 1921) and (*b*) diagnostic doubts raised by the absence of such evacuations may be resolved by blood examinations, with the aid of which it will be possible not only to ascertain the presence or absence of malaria parasites, but also to establish whether the specific gravity of the blood is increased, as ought to be the case in algid cholera patients Moreover as pointed out by Rogers (1952) in malaria patients one finds an increase of the large mononuclears without the total increase of the leucocytes met with in cholera. Pending blood examination the algid malaria patients will be benefited by the administration of saline infusions but it is most essential to start adequate anti malarial treatment with the least possible loss of time.

Parasitic infestations

Referring to the contention of Manson Bahr (1942) that the early stage of trichinosis might be confused with cholera, Strong (1944) stated

"When the adult worms in the small intestine reach sexual activity gastro-intestinal irritation is produced. Abdominal pain, vomiting, severe diarrhoea of the choleraic type may ensue if the infections are severe, with muscular cramps and pain. The presence of eosinophilia and the finding of trichinae would, however establish the diagnosis."

Napier (1946) making a similar statement, pointed to the rarity of trichinosis in India As the present writer noted human infestation with trichinellae is also rather rare in China.

According to Strong (1944) observations in Assam had shown that infestations with the trematode *Gastrodiscus hominis* led to acute intestinal disturbances apt to terminate in death and that, consequently individuals thus affected had sometimes been supposed to suffer from cholera. A proper diagnosis could be made by searching for the ova of the parasites or for the reddish translucent flukes themselves in the faeces of such patients, which were at the same time negative for *V cholerae*

It was also claimed by Das Gupta and co-workers (1944) that a fulminant type of *Giardia* infestation could occasionally lead to violent diarrhoea and other signs of choleraic disease. However as pointed out by the reviewer of the article of these workers in the *Tropical Diseases Bulletin* it was uncertain whether the *Giardia* infestation was the real cause of these disturbances or of the dysentery like syndromes becoming manifest in some other individuals showing evidence of such infestation.

Action of poisons

Various poisonous substances, including the preformed toxin of *Clostridium botulinum* poisonous mushrooms and chemical poisons like antimony arsenic and according to Guttmann (1892) antipyrin as well, have been found to produce clinical syndromes more or less resembling cholera attacks.

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Meningitis Since as noted above cholera affected children may exhibit marked signs of meningismus or similar conditions it is not surprising to find that such patients have sometimes been supposed not to suffer from that disease but from true meningitis On the other hand as once observed by Wilkinson (1943) for instance patients suffering from the latter disease may be sent to the cholera wards Laboratory examination of the stools cerebrospinal fluid or both, must be resorted to in order to differentiate in a reliable manner between cholera with signs of meningismus and essentially meningeal affections

Intra-abdominal haemorrhage It is distressing to note that as exemplified by an observation of Wilkinson a diagnosis of cholera has sometimes been made in the case of women who showed signs of collapse and circulatory failure due to intra abdominal haemorrhage after rupture of an ectopic preg nancy Every possible effort must be made to ensure that such patients or those with other acute abdominal affections, if mistakenly sent to a cholera hospital receive prompt surgical attention Generally speaking, a fully adequate preliminary sifting of the patients admitted to the cholera wards is essential to correct the diagnostic errors made through carelessness ignorance or panicky fear of the infection

PROGNOSIS

It is consoling to note that improvements in the methods of treatment, to which attention will be paid below have led to a marked reduction of the mortality from cholera According to Griesinger (1857) during the early manifestations of the disease in Europe usually about 50%, not rarely approximately 60% and occasionally even a higher percentage of the sufferers succumbed and, as summarized by Rogers (1921) similar or sometimes even considerably higher figures were recorded by the early workers in India. On the contrary gratifyingly low death rates have been reported by several modern observers Thus Mooser and co-workers (1939) as well as Robertson & Pollitzer (1939) stated that, in spite of the havoc generally caused in unoccupied China by a wholesale spread of cholera during the Sino-Japanese hostilities, they had been able to reduce the mortality from this disease in isolation wards established under the auspices of the League of Nations to 9.5% and even to 7 5% Similarly El Ramli (1948) recorded that during the unforeseen calamity of the 1947 cholera outbreak in Egypt which in general caused a mortality of about 50% (see Shousha, 1948) only 83 (12.7%) of his 653 patients who survived longer than 5 hours after admission succumbed to the disease while the mortality among the sufferers coming from the vicinity of the hospital was only 6 6% Tao and colleagues (1948) working after the restoration of peace in China in a permanently established cholera hospital reported a mortality of 6 2%

As pointed out by Strong (1944) botulism may be manifested initially by nausea or vomiting, and occasionally also by diarrhoea, associated with marked prostration but not with fever However in the course of this affection constipation is as a rule present. More important still, besides the manifestation of some signs also met with in cholera, like impairment of the voice and dimness of vision, progressive bulbar paralysis leads to the appearance of palpebral ptosis diplopia, inability to swallow and respiratory paralysis Hence, even apart from the results of stool examination, doubts whether a patient suffers from cholera or from botulism will not persist for long.

The " mycetismus choleriformis " apt to develop after the consumption of poisonous mushrooms, was according to Strong characterized by the appearance of nausea or vomiting and usually profuse diarrhoea, but in contrast to cholera vomiting commenced before the onset of purging and violent abdominal pains became apparent. Apart from the history of the patients a *prima facie* diagnosis of mycetismus could be made through inspection of the stools, which was apt to show the presence of mushroom particles While mushroom poisoning was apt to lead to the appearance of a toxic nephritis with anuria, it was often also responsible for a severe hepatitis with marked jaundice

The signs differentiating arsenic and antimony poisoning from cholera are enumerated in Table XX based on Napier (1946) Guttmann s antipyrin poisoned patient, though otherwise showing signs of a choleraic affection, had normal stools

Stokers cramp

As summarized by Strong (1944) there exists a similarity between the clinical manifestations of cholera and those of stokers cramp a syndrome developing in persons working under conditions of excessive heat and moisture and characterized by the appearance of severe muscular cramps, collapse and sometimes frequent watery stools A similar condition also brought about by excessive sweating and loss of chlorides, has been observed among travellers in hot and dry desert areas

Unless the possibility of cholera can be altogether excluded, the stools of persons showing signs of either of these syndromes must be examined for the presence of *V cholerae* pending the administration of fluids containing adequately large amounts of sodium chloride

Other diseases

The following other diseases, the differentiation of which from cholera has come into question, deserve mention [1]

[1] For a more elaborate list, compiled according to recent experiences in Egypt, see Hassan (1948) Rogers (1952) further mentioned that sometimes clinical similarity could exist between cholera and sunstroke.

Meningitis Since as noted above cholera affected children may exhibit marked signs of meningismus or similar conditions it is not surprising to find that such patients have sometimes been supposed not to suffer from that disease but from true meningitis On the other hand, as once observed by Wilkinson (1943) for instance patients suffering from the latter disease may be sent to the cholera wards Laboratory examination of the stools cerebrospinal fluid or both must be resorted to in order to differentiate in a reliable manner between cholera with signs of meningismus and essentially meningeal affections

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in a total of 2064 patients showing clinical features of the disease, while the death rate among the 687 patients with positive bacteriological findings was as low as 4.6%.

Undeniable though it is that, on account of the then prominent dehydration and circulatory failure, the life of the patients is most endangered during the algid stage of cholera, it is of the utmost prognostic importance to note that only about two-thirds of the total deaths from the disease take place during this initial period of one to two days, while the remainder of the sufferers succumb later even though they have reached the reaction stage. In the experience of most observers, uraemia was the most important cause of these late deaths, hyperpyrexia or other complications, like pneumonia, being less often responsible for the fatal termination of the disease. However Rogers (1921) drew attention to observations made in the case of European cholera patients treated during the period 1895-1906 in Calcutta, among whom 23% succumbed to hyperpyrexia and only 15% to uraemia.

While as will be discussed below certain other factors may be of prognostic importance as far as individual patients or groups of patients are concerned generally speaking, the outcome of cholera depends largely upon the rapidity as well as upon the efficacy of treatment. This point is well illustrated by observations tabulated by El-Ramli as follows

	Days of illness before admission					
	one	two	three	four	more	total
Number of patients	300	168	101	70	38	677
Number of deaths	28	30	26	26	7	117
Death-rate	9.3	17.8	25.7	37.1	18.4	17.3

The great importance of rapid and efficacious treatment depends not only upon the life-saving effect immediately produced through restoration of the fluid balance and circulation, but also upon the rapid restoration of the urine flow which in its turn governs the frequency with which uraemia develops. The validity of this contention is well exemplified by the statement of Rogers (1921) that out of a group of cholera patients admitted at various intervals after onset of the disease,

"only 26.2 per cent. of the uraemic cases were admitted within the first twelve hours of the disease, and no less than 23 or 54.8 per cent., were only brought between one and nine days after the onset, in whom the urine had been suppressed on admission for over twelve hours in 3 for from one to two days in 8, and for two days or more in no less than 12, or over half Suppression of urine for over twenty-four hours is, therefore, of serious prognostic import, which becomes increasingly grave as each day passes without restoration of the renal function "

Besides those mentioned above, the following factors are of prognostic importance in cholera.

Age of patients

As discussed above the prognosis of cholera is markedly more serious in young children and in aged persons Rogers (1921) also concluded from fairly ample statistical material that the outlook was best in adolescents (11 20 years) and that the death rate then rose steadily in each subsequent age group to become maximal in those above 50 years Similarly El Ramli (1948) stated that

"Death rate is lowest among patients of the age group 5-20 years. After 20 it increases with age till it reaches the maximum (50.9 %) after 60 years. The death rate in the group 1 5 years is also high (36.6 %) while in 0-1 year age group it is 11%."

El Ramli s reference to a low mortality in babies less than one year old is rather surprising because, according to earlier observers (see, for instance, Griesinger 1857 and Sticker 1912) cholera, while almost invariably fatal in the newborn claimed a death toll of 80% 90% in the first year of life.

Influence of sex

Notwithstanding a few statements to the contrary it would appear that the sex of the patients exerts no influence on the prognosis of cholera. However as discussed in an earlier part of this chapter in the experience of many observers the occurrence of the disease in pregnant women was fraught with particular danger

Pre-existing abnormal conditions

It is unanimously held that the state of health of individuals contracting cholera infection exerts an important influence on the outcome of the disease the chances of recovery becoming considerably less in persons weakened by pre-existing diseases or by poor nutrition The prognosis has been found to be particularly unfavourable in alcoholics, in opium addicts, in persons with chronic kidney affections, in those with liver complaints (Macleod, 1910) and, as noted by El Ramli (1948) in lunatics.

Influence of vaccination

It is gratifying to note, on the other hand, that previous anti-cholera vaccination, while, in the experience of most workers, not materially influencing the course of the disease, was markedly apt to lower the death rate The earlier evidence available in this respect has been confirmed by observations made during the 1947 Egyptian epidemic when, as stated by Shousha (1948)

"Among 3 648 cases of cholera in eight different fever-hospitals, 396 (*i.e.* 18 7%) were inoculated and 1 721 (*i.e.* 81 1 %) were non-inoculated. The fatality rate was 26.5 % among the inoculated and 42.9 % among those non-inoculated."

Less ample but still more interesting, experience by El Ramli (1948) of cholera mortality in previously vaccinated patients was as follows

Mode of vaccination	*Patients*	*Deaths*	*Mortality rate*
One injection 1-6 days before onset	75	7	9.3
One injection 7 or more days before onset	45	2	4.4
Two injections	38	3	7.9
Total	158	12	7.6

The mortality rate among the non vaccinated was 17.3%.

As El Ramli added the vaccination of patients at the onset of cholera or during the first three days of illness exerted no influence on the course of the disease or on the fatality rate

Long-distance transport

Experience has taught that the chances of recovery under treatment are poor in cholera patients brought to the hospitals from distant localities There can be no doubt that the delay with which the treatment of such sufferers is started largely accounts for the unfavourable therapeutic results. As the same time, however it stands to reason that the hardships these patients undergo during transit also militate against their survival

Character of the clinical manifestations

As aptly stated by Macleod (1910) signs of good or evil omen become observable at every step during the course of cholera. According to him

"*Evil signs* in the order of the stages, are—sudden seizure, early prostration, early stupor quick advent of collapse restlessness, and fighting for breath, failing pulse great depression of temperature prolonged cold stage, hyperpyrexia, severe abdominal pain, blood in vomit and stools, persistent suppression of bile and urine permanent muscular contractions jaundice, lung complications, recurrent purging and vomiting, delayed restoration of body heat, typhoid symptoms, and indications of uraemia or cholo-uraemia, insomnia, and delirium."

Favourable signs, on the other hand, were

"maintenance of pulse during collapse, moderate depression of temperature, early and not excessive reaction, return of colour in the motions, cessation of cramps, restoration of urinary excretion, resumption of warmth and dryness of skin and normal colour of face, quiet breathing, tranquillity sleep"

Macleod added with great reason that, while violent purging and vomiting in the early stage of cholera were not necessarily indicative of a severe seizure their persistence (or one should add, their recurrence) was apt not only to delay convalescence but even to produce fatal exhaustion

True as it is that the majority of deaths from cholera take place during the collapse stage one should beware of undue optimism when one s patients have reached the stage of reaction For, even though at first the condition of the sufferers may thus appear to be favourable, uraemia or other dangerous complications may still become manifest, and even when convalescence has commenced occasional deaths from heart failure may still occur Therefore even if there is reason to hope for a favourable outcome of the disease, one should not be prematurely sure of it.

In addition to a careful and constant general observation of the patients, advantage must be taken of additional methods to assess their condition This is particularly true of stool and urine examinations

The number and the character of the intestinal evacuations must be watched, keeping in mind that early in the disease a sudden cessation of the diarrhoea may be an evil rather than a favourable sign because particularly if combined with the presence of abundant fluid contents in the intestines it is indicative of intestinal paralysis (Sticker 1912) The presence of blood in the evacuations, especially early in the disease, is most inauspicious. On the other hand, as noted by Macleod for instance, (see above) the reappearance of normally coloured motions is reassuring. Constipation in the reaction stage is on the whole less satisfactory than a moderate evacuation of even non-solid stools and should if necessary be combated through the cautious use of microclysters or enemas

A careful and continuous watch over the urine excretion, the successive daily amounts of which should be plotted on charts or graphs, is essential. The specific gravity of the urine should be measured and analyses for the presence of albumin as well as examinations of the sediment for casts, erythrocytes and other cells should be made. While the passing presence of albumin and casts early in the reaction stage is not disquieting, absence of a restoration of the diuresis or its subsequent deterioration are most alarming. A continuously satisfactory urine secretion on the other hand is one of the most reliable and reassuring prognostic signs. To get indications of the presence of acidosis, the reaction of the urine must be watched and tests for acetone must be made

As maintained by Rogers (1952) a drop of the rectal temperature below normal or an excessively high temperature in the rectum associated with coldness of the extremities indicate a most serious condition in collapsed cholera patients. The same is true of the continuous presence of high fever in the reaction stage

Rogers (1921) declared that

> "In the stage of copious evacuations tending to produce collapse the blood-pressure indicating the degree of failure of the circulation, and the specific gravity of the blood, affording a measure of the loss of fluid from the system, furnish the most important information regarding the prognosis."

He maintained, however that a great decrease in the blood pressure was of more serious import than a very high specific gravity of the blood. Be this as it may as far as possible the latter as well as the former should be determined in the early stages of cholera, the more so because, as will be discussed below measurements of the blood specific gravity offer convenient guidance in the proper conduct of the infusion treatment.

Though one must agree with Rogers that a markedly reduced alkalinity of the blood is a *signum mali ominis* it would be most difficult to take clinical advantage of such determinations, when during serious cholera outbreaks numerous patients have to be attended at one and the same time. The same is true of blood urea determinations, highly valued by El Ramli (1948) and of leucocyte counts, the prognostic importance of which was emphasized by Biernacki (1895) and by Rogers (1902)

TREATMENT [1]

A survey of the most voluminous early literature on the treatment of cholera [2] shows that, as Griesinger (1857) sarcastically remarked in their attempts to cure patients suffering from this disease the various workers resorted to almost the whole *materia medica* Thus it came about that diametrically opposed therapeutic methods were recommended, for instance blood letting and blood transfusion laxatives (including even croton oil) and opium or other remedies counteracting the purging, applications of heat or of cold There can be no doubt that many of the early workers, in order to take some kind of therapeutic action against the scourge, resorted without much thought to the remedies they were wont to use However it is consoling to find that, at least as soon as cholera became rampant in Europe rational methods of treatment were proposed side by side with many others which were useless or even harmful for the unfortunate sufferers. Particularly as will be discussed below the history of cholera treatment with intravenous saline infusions, which continue to be the principal means of therapy to date goes back to these early days The same is true of the almost equally important use of alkalis and some still used minor therapeutic methods, for instance, medication with essential oils.

Since both saline infusions and alkali treatment, being insufficiently and inadequately used, did not give the same beneficial results as nowadays, their fundamental importance was not, or at least not fully recognized, and for many decades the treatment of cholera remained in almost the same

[1] In this section, the various treatment schedules and formulae discussed are generally given in the measures indicated by the original authors The metric equivalents of these measures are 1 minim = 0.059 ml; 1 fluid dram = 3.551 ml 1 fluid ounce = 28.41 ml 1 pint = 0.568 l; 1 grain = 0.064 g 1 scruple = 1.296 g; 1 dram = 3.887 g 1 ounce = 28.34 g 1 pound = 0.453 kg

[2] The exhaustive and partly classified list of the early publications on cholera treatment in the third volume of the *Index-Catalogue of the Library of the Surgeon-General's Office United States Army* Washington, D C., 1882, comprises 26 closely printed octavo pages.

confused state as at first. That advanced thinkers nevertheless continued to realize the necessity for a rational instead of a haphazard treatment of the disease is well exemplified by Griesinger s statement that

"As early as the first epidemics ample experiences showed that in the course of the disease, and particularly in its most dangerous stages, nature does more than the physician that all that can be achieved can be attained with simple means, and that excessive medical zeal is as harmful here as in general. We deal with cholera as with typhoid powerless with our therapy to reach the centre of the morbid process and thus to suppress the process, we are mainly restricted to assisting the sufferers through it by doing justice to the most urgent—and, in the case of cholera, almost invariably vital—indications it also seems possible to retard the incipient morbid process and thus to prevent a transition of the still slight initial stage into the stormy and dangerous form of the fully developed illness." [Trans.]

While putting the diagnosis and control of the disease on a rational basis the discovery of the cholera vibrio did not lead to therapeutic progress Attempts were made to suppress the specific morbid process through the administration of various intestinal antiseptics or other drugs or with the aid of therapeutic sera, but neither method gave satisfactory results. Thus real progress commenced only when Rogers (1909a) introducing improved methods of infusion treatment, revived interest in the necessity and eminent usefulness of treating cholera through a restoration of the lost body fluids and salts As noted already this method combined with the judicious administration of alkalis has retained its fundamental value in spite of continued attempts at a specific therapy with the aid, first, of bacteriophages and recently of sulfonamides or antibiotics. Thus, expressing with the aid of modern concepts an opinion essentially rather similar to that of Griesinger Napier (1946) was justified in stating that

"The complete success of efficient biochemical maintenance in a large percentage of cases indicates that, even in those cases in which the natural immunity fails to prevent the establishment of infection, immunity is rapidly developed and soon overcomes the infection. Nevertheless it is obvious that if the infection could be overcome and/or its toxin neutralized earlier the treatment to maintain biochemical balance might be reduced or even omitted in some cases without endangering the life of the patient."

Following Napier it is proposed to deal for the purpose of a detailed discussion of the historically or currently important methods of cholera treatment, first with the various attempts at a specific therapy next with the steps necessary to restore the biochemical equilibrium of the sufferers, and finally with adjuvant therapeutic procedures and management of the patients in general.

Attempts at Specific Therapy

Serotherapy

The history of cholera serotherapy goes back to the year 1894 when Freymuth reported that two out of three patients suffering from the disease

had recovered after they had received subcutaneous injections of cholera convalescent serum in quantities totalling 20-90 ml. Whether these sufferers *also received saline infusions is not mentioned.*

For the convenience of the record it is added that use of this method of treatment was made again by Ukil & Guha Thakurta (1930) who found that better therapeutic results could be obtained if instead of 100 ml of a commercially prepared cholera immune serum, 10-15 ml of cholera convalescent serum were administered by the intravenous route

A review in the *Tropical Diseases Bulletin* (1931) stated that Metz (1930) resorted for therapeutic purposes to " the transfusion of blood from patients convalescent after cholera, called the serum method " Out of 15 cholera sufferers treated in this manner seven recovered, whereas there were only two recoveries among 17 patients treated at the same time in the conventional manner

A further attempt to use human immune serum for the treatment of cholera was made by Ricou & Tran Van Tam (1931) who for this purpose injected healthy group-4 blood donors, free from syphilis, tuberculosis and leprosy subcutaneously with four doses (1 ml up to 4 ml) of cholera vaccine at six days intervals As stated in the *Tropical Diseases Bulletin* (1932) these individuals

" were ready to supply blood after the 2nd injection and received a dose of 1 cc. cholera vaccine intramuscularly before each transfusion. The transfusion procedure used was that of glucose serum and amounts of 150 to 200 cc. were administered daily without any mishap "

However since the cholera patients to whom this treatment could be administered were in a state of profound collapse with anuria, it proved ineffective

According to Takano and co-authors (1926) the problem of cholera serotherapy was studied in Japan during the year 1902. Thus Masuyama (1903) used a cholera-immune serum prepared at the Government Serum Institute for the treatment of 218 patients He found that, " except when practised in the second stage of the illness in youth " serotherapy was not efficacious. Kaya (1903) even claimed that it exerted an untoward effect. Fukuhara (1903) on the contrary recorded that out of a group of 43 cholera patients all seven treated with serum recovered, whereas there were 19 deaths among the 36 controls.

As Takano and his co-workers added, but little therapeutic advantage was afterwards taken of cholera immune serum in Japan, even though its manufacture was continued for laboratory uses and also for the preparation of sensitized vaccines

Strong (1907) recorded that *intravenous administration of the serum of* Brau & Denier (1906) in dosages averaging 300-500 ml gave the following

results in patients suffering from bacteriologically confirmed cholera

	Patients	Recovered	Died	Mortality (%)
Treated with antitoxic serum	15	4	11	73.3
Treated with antimicrobic serum	5	3	2	40 0
Controls	18	5	13	72.2

As far as these figures go the antitoxic serum proved unsatisfactory while owing to the limited number of observations the value of the "anti microbic" serum (manufactured with the aid of live cholera vibrios) was not proven

Fairly ample observations on the efficacy of some of the other cholera immune sera during the 1908-09 outbreaks in Russia, especially at St Petersburg, led to the following noteworthy results

(a) Serum of Kraus (1909)

Observer	Findings
Albanus et al. (1909)	The total mortality of 54 serum-treated cholera patients was 55.5 % (being 58.8 % in the 17 injected subcutaneously and 54 % in those receiving up to 120 ml intravenously) whereas 48.5 % of 490 controls succumbed. It was true that 84.3 % of the severely affected controls died, but—in contrast to the serum-treated patients—most of them had received no saline. Results of serum treatment in two further series of 10 and 41 cholera patients were still more unsatisfactory
Hundögger (1909)	The mortality in 38 patients treated with saline and with intravenous doses of 100 ml of serum was 52.6 % as against 48 % in 156 saline-treated controls. Serum administration did not prevent the development of uraemia.
Jegunoff (1909)	Recorded fatality rates of (*a*) 50 % in 12 serum- and saline-treated patients (*b*) 52.9 % in the whole control group of 34 and (*c*) 75 % in the severely or moderately affected controls. However as Jegunoff maintained with great reason, owing to the small number of observations no proof of the efficacy of Kraus's serum had been obtained.

Note Berthenson (1909) in a report summarizing the results of various methods of cholera serotherapy stated that Kraus's serum had proved unsatisfactory

(b) Serum of Schurupow (1909)

Observer	Findings
Berdnikoff (1909)	Used Schurupow's serum intravenously in amounts of 40-50 ml mixed with 2 3 l of normal saline to treat 49 patients. In the case of a group of 10 patients thus treated the mortality was reduced to 36 % as against 70 % in the controls. But administration of other serum lots to 39 patients proved unsatisfactory

Observer	Findings				
Stühlern (1909)	Recorded the following results				
	Mode of treatment	*Treated*	*Cured*	*Died*	*Mortality (%)*
	100-200 ml doses of serum intravenously 1 3 times daily with large amounts of normal saline and 50-90 ml of serum subcutaneously 1 2 times daily (maximal total serum dose = 1390 ml)	187	131	56	29 9
	Intravenous saline infusions	228	131	97	42.5
	Subcutaneous saline infusions	742	335	407	54 8
Haller (1911)	The mortality in a group of 20 severely affected cholera patients receiving daily intravenous doses of a maximum of 220 ml of the serum was reduced to the satisfactory level of 40 %. However serious signs of anaphylaxis became manifest in several of the patients and deaths due to this cause occurred twice				

Note As stated by Berthenson (1909) some authorities, in view of the varying results obtained by different workers, denied the efficacy of Schurupow s serum. Still Stühlern s results are impressive, though worse than those now obtainable with infusion treatment alone.

(c) Serum of Salimbeni (1908)

Salimbeni (1910) recorded the following results of subcutaneous administration of his serum in minimal doses of 100 ml mixed with normal saline and followed, if necessary by intravenous injections of 50-100 ml of serum (maximal total serum dosage = 350 ml)

Type of cholera	*Treated*	*Recovered*	*Died*	*Mortality (%)*
Most severe	19	10	9	47 4
Severe	10	9	1	10.0
Less severe or slight	13	13	0	—
Total	42	32	10	23 8

However Berthenson (1909) reported a mortality of 62 % in 94 cholera patients who had received subcutaneous injections of Salimbeni s serum.

(d) Berne serum

As reported by Kolle (1909) the cholera immune serum prepared in his laboratory (see also Carrière & Tomarkin, 1910) had been used at St. Petersburg, together with saline, in partly subcutaneously and partly intravenously administered doses of 50-120 ml to treat 22 patients, of whom five (22.7 %) died.

As stated by Savas (1914) further and, in part, fairly ample observations on the efficacy of cholera immune sera obtained from various places (Institut Pasteur in Paris Kolle s laboratory in Berne Vienna and Dresden) had been made in 1913 during the Balkan wars in Greece These observations included findings made at Salonika by Livierato (see also Livierato 1915) who administered Berne serum intravenously in several doses of 40-120 ml

to 61 severely affected cholera patients in combination with hypertonic saline infusions and cardiac stimulants Whereas 17 controls treated only with the latter two remedies, all succumbed, the mortality among the serum treated sufferers was 55 7%

Commenting upon this and also the other observations made at that time in Greece Savas held that

"Timely intravenous injections of larger amounts of cholera serum, particularly in combination with normal or hypertonic saline infusions, were often successful and are therefore to be warmly recommended for the treatment of cholera." [Trans.]

As summarized by Hetsch (1928) strikingly little use of cholera serotherapy was made during the First World War the only noteworthy findings being those of Bujwid & Arzt (1914) who briefly recorded a mortality of 15% in 40 patients treated with a therapeutic serum manufactured at Cracow as against a fatality rate of 26% in the controls

Generally speaking, Hetsch admitted that cholera serotherapy had not given uniformly good results, but cautiously added that it would be rash to consider the prospects of this method of treatment unfavourable. It is significant, however that Kraus (1929) formerly an ardent advocate of cholera serotherapy came to the conclusion that the immune sera, though efficacious in experimentally infected animals, had not given satisfactory results (*kein günstiges Ergebniss*) in man

As alluded to in Chapter 6 attempts to revive interest in cholera serotherapy have been made by Ghosh (1935 1936) As this worker stated in a preliminary note (1935) he obtained the following results when intraperitoneally administering a concentrated serum produced by him through immunization of horses with filtrates of 18-hour-old *V cholerae* broth cultures, in doses of 20 ml or of 30-40 ml in combination with saline infusions

Method of treatment	*Patients*	*Recovered*	*Died*	*Fatality rate*
Serum and saline	198	158	40	20.2
Saline only	211	138	73	34 6

Note The mortality percentage in the 32 patients who had received 30-40 ml of the serum was 12.5 as against 26.3 in the corresponding control group of 57 patients

Further results obtained by Ghosh (1936) when intraperitoneally administering 70-80 ml of unconcentrated serum to 47 patients also treated either with only one intravenous saline infusion on admission or additionally with subcutaneous saline injections, were as follows

Method of treatment	*Patients*	*Recovered*	*Died*	*Fatality rate*
Serum and saline	47	42	5	10.6
Saline only	170	135	35	20.6

Note The mortality rate in 26 serum-treated patients with a blood specific gravity of 1.064 or more was 11.5 % as against 25 % in the corresponding control group of 44 patients. Ghosh also stressed that the serum-treated patients never fell into a uraemic state.

It is undeniable that Ghosh as well as some of the workers before him obtained fairly satisfactory results with cholera serotherapy At the same time however the present writer is in full agreement with the statement of Strong (1944) that

" no one has reported a lower mortality in a large series of cases treated with serum than has been obtained by careful treatment with intravenous injections of saline and alkaline solutions."

Before dealing with the modern phase of cholera therapy attention has to be paid to the employment of three drugs which, though not of a specific nature were thought capable of suppressing the development of severe attacks or of counteracting the *V cholerae* toxin.

Treatment with essential oils

As can be gathered from the reference list in the 1882 volume of the *Index Catalogue of the Library of the Surgeon-General's Office* mentioned above, medication with cajeput oil, one of the ingredients of the essential oils mixture afterwards used for the treatment of cholera had been advocated by some workers in Europe e.g. by Tierney and by Bushell in Great Britain, as early as 1831 It is probable that even before that time this therapeutic method had been resorted to in India. It is certain that both Sealy (1922) and Tomb (1923 1926) when again drawing the attention of the medical profession to this method of therapy pointed out that it had been well known and used with apparent success by laymen in India for many years.

Sealy (1922) worked mainly with a " pro-diarrhoea mixture " containing the volatile oils of cajeput, aniseed and juniper besides tincture of cinnamon and other ingredients administered in doses of 40 minims (containing 12 15 minims of the essential oils) at intervals of half an hour However regardless of whether this mixture of essential oils or oils of cloves or of cinnamon alone were used, he found that the early administration of these drugs to cholera patients invariably led to a suppression of the attacks. Since the essential oils were insoluble and inadsorbable in the stomach Sealy maintained that their therapeutic efficacy depended upon a bactericidal effect exerted on the cholera vibrios in the intestines.

The mixture of essential oils initially recommended by Tomb (1923) was prepared with the oils of cloves, cajeput and juniper However in order to obtain a less pungent, and therefore more palatable, preparation, Tomb (1926) advocated the following new formula

	Minims
Ol. anisi	5
Ol. cajeputi	5
Ol. juniperi	5
Acid. sulfuricum aromaticum	15
Spirit. aetheris	30

Note For preparation in bulk 3 pounds of the sulfuric acid could be mixed with 6 pounds of Spirit. aetheris, and then 1 pound quantities of the essential oils be incorporated.

Dosages 1 dram every half-hour or ½ dram every quarter-hour in water until 1 ounce was given, then 1 dram every hour until complete recovery For contacts 1 dram in water one or two times daily for 1 2 days or as long as contact with patients continued.

In this paper as well as in two other papers published in 1924 and 1929 Tomb emphasized the great value of the essential oils both in the treatment and in the prevention of cholera

Regarding the curative value of the essential oils, Tomb (1924) furnished the following statistics

Method of treatment	*Patients*	*Recovered*	*Died*	*Mortality (%)*
Essential oils	78*	62	16	20.5
"Cholera mixtures"	217	98	119	54 8
Untreated	50	2	48	96.0

* The mortality among 60 of these patients who ill for an average of 7 hours, were not in a collapsed condition was 5 %, as against 72 % in 18 patients who having been ill for an average of 12 hours before commencement of treatment, were collapsed.

Further observations made by Tomb regarding the efficacy of the essential oils in the treatment of cholera are well exemplified by the following tabulation culled from his 1929 paper

Method of treatment	*Not collapsed before treatment*			*Collapsed when treatment was started*		
	patient	*deaths*	*mortality (%)*	*patients*	*deaths*	*mortality (%)*
"Cholera mixtures" *	245	45	18 4	263	192	73 0
Saline infusions	11	2	18.2	51	24	47.0
Homoeopathic **	38	5	13.2	30	22	73.3
Essential oils	41	3	7.3	44	21	47 7
Untreated	—	—	—	102	95	93 1

* Containing chlorodyne or other opium preparations.

** Consisting mainly of administration of spirits of camphor

Referring to the observations he had made regarding the prophylactic value of the essential oils, Tomb (1926) summarized that there were less than 1 % of cholera infections among several thousands of cholera contacts (i.e., house-mates of patients showing the clinical features of cholera) who had been dosed once or—if nursing the sufferers—twice daily as long as the risk of infection continued He added that three daily medications with essential oils practically precluded infection

The great value of treatment with essential oils in the early stage of cholera was endorsed by a number of subsequent workers, for instance by Yui (1925), Bharati (1926) Lieo (1926) Cannon (1927) Morison Rice & Haythornthwaite (1934) However, the usefulness of this therapeutic method has been questioned by some of the modern compilers particularly by Napier (1946) who maintained that in resorting to it one merely made a "gesture of despair"

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to bring about an improvement of the pulse, rectal introduction of heavy suspensions of kaolin was commenced at once and had a marked effect in preventing the further loss of fluid On the return of consciousness and during the introduction of the saline kaolin was given by the mouth and was usually retained

Walker added that there was not a single death among 75 cholera patients treated in this manner, even though many of them arrived in the hospital in a condition of extreme collapse He established through laboratory tests that kaolin exerted no bactericidal action in *V cholerae* cultures but found that treatment of the filtrates of such cultures with kaolin rendered them non fatal for rabbits [1]

Comparing different methods of cholera treatment Chatterjee (1924) recorded the following results

Method of treatment	*Number treated*	*Stage of the disease*	*Cured*	*Died*	*Remarks*
Kaolin	6	In early stage	3	3	2 drams in water every half-hour for 4 hours
Kaolin and permanganate	6	2 in early 3 in late stage	5	1	Every half-hour one 2-grain pill of potassium permanganate and 1 dram of kaolin in water
Essential oils	6	In early stage	2	4	
Essential oils and permanganate	6	2 in early 1 in late stage	3	3	Pink solution of potassium permanganate given as drink

* Cured patients only

On account of these findings Chatterjee was in favour of combined treatment with kaolin and potassium permanganate but admitted the necessity of further observations.

In the experience of Ghosh Dastidar (1925) kaolin was useful for the treatment of cholera patients in the early stage of the disease or suffering from mild attacks

Tomb (1926) while admitting that thorough treatment with kaolin reduced the mortality from cholera to 25%, noted that this therapy was disliked by the patients and was unsuitable for field work on account of the difficulty of transporting the required bulky supplies of the drug. It was perhaps on account of such extrinsic difficulties, which were also experienced by the present writer that Strong (1944) Napier (1946) and Manson Bahr (1954) viewed the method of treating cholera patients with kaolin with great disfavour

The proposal of Groák (1915) and of Adler (1916) to use animal charcoal for the treatment of cholera instead of kaolin, seems to have won no approval

[1] Further laboratory tests by Dhar (1930) showed that addition of large quantities of kaolin to cholera cultures exerted a hampering effect on the growth of the organisms which, however was due merely to physical causes. Dhar & Sen (1928) had previously shown that the rate of absorption of killed cholera vibrios by kaolin was higher than that of killed typhoid bacilli or *E coli*

To contradict this statement is difficult. It is true that, in agreement with the assertions made by laymen in India, the present writer met in China with observant lay people including Roman Catholic Sisters with long experience in dispensary work, who felt convinced of the therapeutic value of preparations similar to the essential oils mixtures used in India. However it is an open question how many of the patients thus successfully treated really suffered from cholera and not from simple gastro-enteritis. Moreover even if infected with *V cholerae* the patients in question might have had slight cholera attacks, from which they would have recovered regardless of whether they had been treated with this or any other drug.

Kaolin

Though Brassier in 1831 advised treating cholera patients with *charbon végétal* (wood charcoal), it appears that the use of kaolin, which exerts a similar adsorbent action in the intestines, was recommended for the same purpose only in the twentieth century (Stumpf 1906, 1914). Both Stumpf and Kuhne (1918), who had worked together with him in Serbia, reported enthusiastically on the efficacy of kaolin in the treatment of cholera, the latter stating that with the aid of this drug alone it had been possible to reduce the mortality from the disease from 45% to 2% 3%.

While Stumpf (1914) recommended the use of kaolin suspensions of a strength of 50%, Kuhne s practice was to mix equal volumes of the drug and water (e.g. by putting 100 g of kaolin into 0.25 l of water) and to give one glassful of the suspension every half hour or hour thus administering up to 200 g of kaolin during the first 12 hours of treatment During the next 12 hours and the following day several glasses were given according to the condition of the patient. During the first 18 hours of treatment the sufferers were not permitted to take other drinks or any food.

Further favourable results with kaolin were recorded not only by some of the medical men attending cholera patients during the First World War in Austria and Germany (see, for example Arneth 1916) but also by some workers in China and India

Braadfladt (1920) reporting on the treatment of 100 such patients recorded only one death among the 35 sufferers (15 of them with severe attacks) treated with kaolin alone, as against seven deaths in 24 patients (20 with cholera gravis) also receiving hypertonic saline infusions, and nine deaths in 41 patients (32 with cholera gravis) given only saline infusions

Deviating from the recommendations of Stumpf and Kuhne Walker (1921) encouraged his patients to drink as much 50% kaolin suspensions as possible, withholding at the same time all food for 18-24 hours and he also resorted to rectal lavage with suitably diluted suspensions. He stated that under this regime

" In the lighter cases 12 hours saw the cessation of diarrhoea and 24 to 36 hours the passage of urine. In the most critical cases, which required the introduction of saline

Favourable results with potassium permanganate were also recorded by some subsequent workers

Thus Goéré (1913) found this drug effective for the treatment of cholera carriers Frendl (1914) using it in a 0 1/1000 dilution apparently without concomitant saline infusions, claimed that all but the too severely affected of 326 cholera patients were cured

Maddock (1915) working under conditions in which but little use could be made of saline infusions, recorded a mortality of 35 6% in 4574 cholera patients treated with potassium permanganate pills according to Roger s scheme as against a fatality rate of 51 6% in 11 599 not so treated the corresponding death rates in 483 patients under the supervision of the dispensary staffs were 21 98% and 60% Further as noted above Chatterjee (1924) administering various forms of treatment to a small group of cholera patients, found the combined use of potassium permanganate pills and of essential oils more effective than that of the latter alone.

Unfortunately, however further observations have failed to endorse the favourable experiences recorded above It has first to be noted in this connexion that Pasricha and co-workers (1939) though finding a slightly higher rate of recoveries in patients receiving potassium permanganate pills as well as saline infusions than in a calomel-treated group, noted that the former vomited longer and recovered more slowly [1]

Tomb (1926) declared that potassium permanganate treatment of cholera patients was valueless and this adverse opinion has been shared more recently by Strong (1944) and by Napier (1946) who made the following statement

> "Potassium permanganate has been most disappointing in the writer's experience, and, in the large doses advocated it appears to cause gastro-intestinal irritation very frequently It seems very questionable whether its *in-vitro* toxin-oxidizing properties are reproduced *in vivo*"

Bacteriophage treatment

Noteworthy statements made in support of the therapeutic efficacy of bacteriophages in cholera may be summarized as follows

Recording the results of trials commenced in 1926 in the Punjab d Hérelle and colleagues (1930) stated that they had usually resorted to the oral administration of choleraphages, administering—without using any other kind of treatment—an initial dose of 2 ml in 10 ml of cold water and leaving with the patients a further amount of 4 ml in half a cup of water for gradual consumption during the following three hours if necessary this medication with 6 ml of phage was repeated on the next day

Out of 74 patients treated in this manner only six died whereas there were 78 deaths among 124 controls treated either according to native methods

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[1] Pasricha and his colleagues maintained that additional treatment with essential oils, while giving a higher recovery rate than that of the calomel- or permanganate-treated patients, also delayed recovery

Potassium permanganate

It is curious to find that Wienkowski (1873) apparently the first to use potassium permanganate for cholera treatment, did so in the hope of destroying the fungi (*Pilze*) causing this disease. While claiming success he stressed the necessity of also administering cardiac stimulants to the sufferers.

As exhaustively described by Rogers in 1910 and again in his book, *Bowel Diseases in the Tropics* (1921) he started using permanganate com pounds, in addition to hypertonic saline infusions, for the treatment of cholera in 1909 He resorted to this combined therapy on the assumption that, on account of their oxidizing action the permanganate compounds were capable of destroying the cholera toxin in the intestines.

As Rogers explained, the simplest plan was to administer these drugs (preferably the less astringent calcium permanganate) in the form of drinks, but it was found to be more effective to give them in the form of pills so coated that they dissolved only in the alkaline contents of the intestines the non hygroscopic potassium permanganate was preferable for this purpose. The formula recommended by Rogers was

Finely powdered potassium permanganate	2 grains
Kaolin and vaselin	*Quantum sufficit*

Coat with salol one part and sandarach varnish five parts, or with keratin.

As stressed by Rogers, it was important to ascertain that pills which had been stored for some time were still soluble in 1% alkaline solutions, since *otherwise they might pass through the bowel unchanged.*[1]

The manner of administration of the pills was according to Rogers (1921) as follows

> "Immediately on admission one or two pills containing 2 grains of the salt are given every quarter of an hour for two hours and then one or two pills every half hour any pills which are rejected by the stomach are replaced without delay They are continued until the stools become green and less copious this usually occurs in about twelve to twenty-four hours with this treatment. In mild cases it will suffice to continue the pills during each alternate four-hourly period At the beginning of the second twenty-four hours, eight more pills are given within four hours. In severe cases this is again repeated at the beginning of the third day to prevent a relapse. Nothing but barley water should be given during this treatment "

Rogers (1921) illustrated the value of the permanganates in the treat ment of cholera in a table, from which it can be gathered that 74 1% of 858 patients given these drugs as well as hypertonic saline infusions recovered, as against 67 4 / recoveries in 294 patients receiving only the latter

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[1] This was actually observed in field work by Bharati (1926), who used such pills in combination with essential oils for the treatment of 18 cholera patients, over 80% of whom recovered, even though only six had also been given saline infusions.

		Treated with bacteriophage on 1st day of illness	Treated with bacteriophage on 2nd day of illness	Control
Outbreak at Sibsagar	Recovered	53	42	207
	Died	15	14	152
	Mortality ( )	22.0	25 0	42 3
Outbreak in Darrang villages	Recovered	20	17	23
	Died	16	14	82 *
	Mortality ( )	44 4	45.2	78 1

Exclusive of the patients dying within 24 hours of admission.

In the second article Morison Rice & Choudhury furnished *inter alia* the following interesting statistics

(a) Results of bacteriophage treatment in Assam 1928 1933

	Recovered	Died	Total	Mortality ( )
Treated with bacteriophage	799	233	1032	22.6
Treated by other methods or untreated	1047	1364	2411	56.6

(b) Comparison of the results of standard treatment in Calcutta and of treatment in Assam

	Treatment	Patients	Deaths	Mortality (%)
Calcutta	Saline and potassium permanganate			
	1st series	659	103	15 6
	2nd series	91	14	15 4
	Saline, potassium permanganate and atropine	97	8	8.2
Assam	Phage on 1st day of illness	482	46	9.5
	Essential oils	400	95	23 7
	Untreated	495	208	42.0

Further work by Pandit and colleagues (1936) during a cholera outbreak in Assam yielded the following results

Method of treatment	Patients	Death	Fatality rate (%)
Bacteriophage only	279	93	33.33 *
Essential oils	66	24	36.4
None	400	286	71 5 **

* The mortality in 55 phage-treated patients who had been vaccinated against cholera was 27 7 %

* Half of the 12 untreated but cholera vaccinated individuals died

Working at Chandernagore India, Boulnois (1936) obtained the following results with bacteriophage treatment commenced within 12 hours after the onset of cholera

Method of treatment	Patients	Deaths	Mortality (%)
Bacteriophage only	19	5	26.3
Bacteriophage + hypertonic saline	37	7	18 9
None	21	13	62.0

or with essential oils. The sooner was phage administration started, the better were the results, no fatality being observed among the 26 patients in whom the treatment had been commenced six hours after the onset of illness

Subcutaneous administration of 1 ml or 2 ml doses of choleraphages in combination with oral treatment gave most disappointing results and was therefore given up after a few trials. This, however was not in agreement with subsequent observations, Ross and co-workers (1928), for instance recording only two fatalities in 16 cholera patients who had received on admission an injection of 0.25 ml of specific phages as well as 2 ml orally Further as will be discussed below Asheshov and colleagues (1931) recommended combining intravenous injection of choleraphages in maximal doses of 5 ml with oral administration

Referring to initial experiences in Assam, Morison & Vardon (1929) recorded a mortality of 29% in 31 phage treated cholera patients (most of whom had received no saline infusions) as against a mortality of 75.9% in 29 controls Again, early commencement of the bacteriophage therapy gave comparatively the best results.

Using, like Morison & Vardon a mixture of cholera and dysentery phages, Morison & co-workers (1930) observed recovery of 58 out of 65 patients (mortality 10 8%) whereas the fatality rate among 78 controls was 80 8%. Only five of the phage-treated patients had also been given saline infusions.

Asheshov Khan & Lahiri (1931) dealing exhaustively with the problem of cholera treatment, stressed the necessity of combining phage administration with saline infusions, because the bacteriophages, though apt to destroy living cholera vibrios, were incapable either of neutralizing the cholera toxins or of exerting an influence on the lesions produced by the latter or by the organisms themselves Asheshov and his colleagues advised oral administration of the choleraphages in dram doses given every half hour during the first 16 hours of treatment and followed by medication with a total of 50 ml given by the same route on each of the next two days Apart from this therapy bacteriophage doses not exceeding 5 ml could be given intravenously together with the saline infusions. Excluding the sufferers who died within two hours of admission the mortality among 140 bacteriologically confirmed cholera patients treated in this manner was only 2.8%, whereas 20 8 / of a corresponding control group of 24 patients succumbed. Evaluating these statistics one cannot help noticing the disproportion of the two groups of patients in question

Two further reports on the bacteriophage treatment of cholera patients were published by Morison and his co-workers in 1934 In the first of these articles, by Morison, Rice & Haythornthwaite, the following results were recorded

Souchard (1930) treating 26 cholera patients who had been ill for at least seven hours, according to the method of d Hérelle and colleagues, found that out of seven patients treated only with bacteriophage six (i e, 85 7%) died while the fatality rate in 20 sufferers receiving also standard treatment was 90%. Ten of the phage treated patients succumbed after a more prolonged illness to uraemia the development of which had thus not been prevented by this therapy However, Souchard was of the opinion that better results might be obtainable, if it were commenced within two to three hours of the onset of the disease—a desideratum which it would usually be most difficult to fulfil

In the course of bacteriophage studies in Madras Presidency (now Madras State) Raja (1934) was able to observe two groups of 36 and 33 cholera patients treated respectively with bacteriophages and with pro-diarrhoea mixture As far as these limited experiences went, they indicated no significant differences between the results of these two methods of treatment. Thus, as Raja put it, it had not been shown that the one was more useful than the other

Pandit & Rice (1936) recorded that during a cholera outbreak in Mon dair village in Assam 27 out of 55 bacteriophage treated patients had died their mortality being thus higher than that of the few controls They added that similarly disappointing results had also been obtained during a 1934 epidemic in Nowgong district, when 16 out of 22 phage-treated cholera patients had succumbed. The explanations which Pandit & Rice offered to account for these failures cannot be considered satisfactory

Even those commentators who did not altogether deny the efficacy of bacteriophage treatment in cholera were as a rule more or less sceptical regarding its value Thus as quoted by Pandit (1915) an *ad hoc* committee appointed by the Scientific Advisory Board of the Indian Research Fund Association in 1934 declared in regard to the therapy of cholera that

"while Morison s work showed that bacteriophage treatment was far better than no treatment at all, there was no conclusive evidence that it was better than any other recog nized treatment"

More recently Burrows (1948) while admitting that "phage seems to have some small therapeutic efficiency as measured by reduction in case fatality rates" maintained that the results of this therapy had not been strikingly successful

Shattuck (1951) went even further by stating that, since clear evidence for the efficacy of phage therapy in cholera was lacking, its use was not to be recommended

On the other hand Raynal (1934) and more recently Napier (1946) maintaining that bacteriophage treatment had given encouraging results, were in favour of its further use, but both stressed the necessity that, as Napier put it a "good" bacteriophage, effective against the local strains

Reporting on a series of observations in the Campbell Hospital Calcutta, Pasricha and colleagues (1936) stated that

(*a*) The mortality in 684 phage treated patients showing clinical features of cholera was 13 4% as against 16 6% in a control group of 685 patients.

(*b*) However bacteriophage treatment appeared to exert a significant effect only on those patients in whose stools cholera vibrios had been found, but not on those from whom only cholera like vibrios or no vibrios had been isolated

(*c*) The incidence of uraemia appeared to be markedly reduced in the phage-treated series.

In a further study Pasricha and co-workers (1939) recorded that administration of various other forms of treatment to cholera patients also receiving saline infusions and the necessary adjuvant medication had given the following results

	Patients	*Deaths*	*Fatality rate* (%)*
Calomel (1/8 *grain every half* hour for maximum of 4 hours)	75	9	12.0
Potassium permanganate	37	4	10.8
Essential oils	46	4	8 7
Cholera phage	43	1	2.3
Sulfapyridine	43	4	9.3

* Excluding those dying within 3 hours of admission as well as those under 6 or over 50 years old.

Referring to observations made in Bihar, India, Mitra (1939) maintained that good results could be obtained by using bacteriophages alone in the early stages of cholera, but that in the presence of collapse and dehydration supplementary treatment with saline infusions was indispensable An analogous conclusion was reached by Misra (1944)

The often enthusiastically expressed belief of the above-quoted authors in the efficacy of cholera treatment with bacteriophages has not been shared by other observers, who obtained disappointing results with this therapy

In order to deal with these unfavourable experiences mention has to be made first of a most noteworthy study by Taylor and colleagues (1930) who making observations on a series of 33 cholera patients, found that the mortality in a group of 14 phage treated sufferers was 57 % as against a fatality rate of 53% in the 19 controls Of almost equal, if not greater importance was that in most of the recovering patients—regardless of whether or not they had been phage treated—there was "no evidence of bacteriophage in the stools active against the patient s own vibrio" In contrast to the assertions of d Hérelle, Malone & Lahiri (1928), Taylor and his colleagues concluded that

"bacteriophage is not an essential agent of recovery and that its administration is not an effective measure in the class of case dealt with."

Souchard (1930) treating 26 cholera patients who had been ill for at least seven hours according to the method of d'Hérelle and colleagues found that out of seven patients treated only with bacteriophage six (i e 85 7%) died while the fatality rate in 20 sufferers receiving also standard treatment was 90%. Ten of the phage treated patients succumbed after a more prolonged illness to uraemia, the development of which had thus not been prevented by this therapy However Souchard was of the opinion that better results might be obtainable, if it were commenced within two to three hours of the onset of the disease—a desideratum which it would usually be most difficult to fulfil

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> while Morison's work showed that bacteriophage treatment was far better than no treatment at all, there was no conclusive evidence that it was better than any other recognized treatment "

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On the other hand, Raynal (1934) and more recently Napier (1946) maintaining that bacteriophage treatment had given encouraging results, were in favour of its further use but both stressed the necessity that, as Napier put it, a " good " bacteriophage, effective against the local strains

of *V cholerae* be used in the various epidemics However even if one were prepared to share the views held by these workers regarding the usefulness of bacteriophage therapy in cholera, one must realize that the extrinsic difficulties of applying it efficiently on a large scale are well high insurmountable Under these circumstances it is not surprising to find that, with the possible exception of Assam (see Pandit, 1951) this method of cholera therapy which attracted so much attention for a time is hardly used any more

Sulfonamides

As far as could be established, Pasricha and co-workers (1939) were the first to attempt treatment of cholera patients with sulfonamides, using for this purpose a proprietary brand of sulfapyridine in a dosage of 2 tablets (presumably of 0 5 g each) three times daily for a maximum of four days Results of this and other methods of treatment, invariably administered in combination with saline infusions have been shown in tabular form on page 764

Before recording the results obtained by numerous subsequent workers with various other sulfonamides in the treatment of cholera patients, it is necessary to deal briefly with important laboratory studies made in order to elucidate the efficacy of this therapy

Rao & Ganapathi (1941) recorded that intragastric administration of 3-mg doses of sulfanilamide sulfapyridine or sulfathiazole to mice, which had been infected immediately before with virulent cholera vibrios, and repetition of this therapy after 10 hours, did not avert the death of the animals. It was found, however that sulfathiazole possessed a marked bacteriostatic effect *in vitro* and that this action was reversed by the addition of para aminobenzoic acid, even though the latter compound exerted no perceptive growth stimulating effect on *V cholerae*

Further studies by Griffitts (1942) showed that, in addition to sulfathiazole sulfadiazine and sulfanilamide also inhibited the growth of cholera vibrios *in vitro* The first two drugs, given subcutaneously or intragastrically were also found effective for the treatment of white mice previously infected intraperitoneally with lethal doses of *V cholerae* in mucin Identical results were obtained with intragastric administration of sulfaguanidine or succinyl sulfathiazole.

Sadusk & Oswald (1943) investigating the bacteriostatic action of various sulfonamides on *V cholerae* found that sulfathiazole exerted the greatest effect in this direction, being followed in order of decreasing efficiency by sulfadiazine sulfaguanidine [1] and sulfanilamide Pointing out that sulfathiazole might be unsuitable for cholera treatment on account of its ready

[1] A bactericidal effect of high concentrations of sulfaguanidine on cholera vibrios had been demonstrated previously by Marshall and co-workers (1940)

absorption from the digestive tract, Sadusk & Oswald recommended the relatively little absorbable sulfaguanidine for this purpose Succinyl sulfathiazole seemed also deserving of attention but its rate of hydrolysis was still uncertain

Performing a few *in vitro* tests Gupta and co-workers (1945) felt entitled to confirm that sulfaguanidine exerted a bactericidal as well as a bacteriostatic action on *V cholerae*

In an article published in 1948 Bhatnagar and colleagues claimed that (*a*) a new sulfa compound being a condensation product of sulfathiazole and of formaldehyde exerted *in vivo* a marked bacteriostatic and bactericidal effect on *V cholerae* and (*b*) administered parenterally to mice the compound offered "100% protection against septicaemia resulting from intraperitoneal cholera infection" Experiments made with oral administration of the drug did not give satisfactory results

In an article dealing with the therapeutic use of phthalylsulfacetimide during the 1947 Egyptian cholera outbreak (see below) Seneca & Henderson (1949a) stated that in addition to exerting a bactericidal action on *V cholerae* this drug

"has the remarkable property of being absorbed by diffusion into the several layers of the intestinal wall, yet being unabsorbable in the sense that blood concentrations of the drug cannot be detected in man following therapeutic dosage"

As stated in an exhaustive summary in the *Tropical Diseases Bulletin* (1950) Collier and his assistants (1949) recommended that in order to arrive at an estimation of the efficacy of sulfonamides and other drugs for the chemotherapy of cholera, attention had to be paid to the following criteria (*a*) vibriostatic activity *in vitro* (*b*) solubility (*c*) toxicity (*d*) retention in the mobile contents of the alimentary canal, (*e*) activity in the alimentary canal and (*f*) stability in the alimentary canal

Using methods based on these criteria for an investigation of the properties of sulfaguanidine and the compound introduced by Bhatnagar and co-workers (see above) Collier and his assistants came to the conclusion that the latter drug appeared to be more promising for the treatment of cholera than sulfaguanidine but that "its inability to deal with very large numbers of organisms suggests that its value may be limited"

It is not proposed to refer in detail to further studies on the chemotherapy of cholera published by Collier and his co-workers in the 1950 and 1951 volumes of the *Annals of Tropical Medicine and Parasitology* because so far the compounds tested by them in the laboratory have not been used for the practical purposes of cholera treatment

In the course of his studies on cholera immunity Burrows (1953) experimented with sulfathiazole, sodium sulfadiazine sulfadiazine and sulfaguanidine giving each of these drugs intragastrically to a separate group of six guinea pigs in an initial dose of 250 mg and a second dose of

125 mg on the day prior to infection, and administering two doses of the latter size on the following day before resorting to oral infection (made according to the method described in Chapter 6) Daily examinations of the faeces of the animals showed that the two more effective sulfonamides used,

" sulfadiazine and sulfaguanidine, appeared to reduce both the total numbers of bacteria and the percent of cholera vibrios, i.e., the cholera vibrio was differentially affected, and to shorten the duration of the infection. The effect of these drugs on the infection, then, was at least superficially closely similar to that of passive immunization, so much so that data on vibrio excretion are practically interchangeable "

Sulfathiazole proved to be less effective, and sodium sulfadiazine had practically no effect.

Taking advantage of their effective method of producing, through intestinal injection of massive doses of *V cholerae* in young rabbits, a disease which clinically and anatomically closely resembled human cholera, Dutta & Habbu (1955) tested the therapeutic efficacy of some drugs including sulfaguanidine and formosulfathiazole, the compound introduced by Bhatnagar and co-workers (see above) Results obtained with these two sulfonamides are shown in the following tabulation

Time of administration (hours)	*Sulfaguanidine*		*Formosulfathiazole*	
	mean survival time (hours)	*mortality*	*mean survival time (hours)*	*mortality*
1 hour before infection	—	0/5	—	0/7
8 hours after infection	32.0	5/9	30.3	8/8
16 hours after infection	33 3	4/4	—	—

* Ratio of animals which died to animals infected

Note The mortality of the control rabbits, infected like the treated animals with 10 000 *V cholerae* per 100 g of body-weight, was invariably 100 %.

Thus while formosulfathiazole was effective only if administered before infection, sulfaguanidine showed some curative value eight hours, but not 16 hours, after the animals had become infected

Further noteworthy results obtained when treating cholera patients with various sulfonamides were as follows

Sulfaguanidine

Reporting on the original use of sulfaguanidine, Chopra and colleagues (1941) stated that

(*a*) The mortality in a series of 218 patients showing the clinical features of cholera and given sulfaguanidine in an initial dose of 1 g, followed by six hourly doses of 0 5 g for three days, in combination with intravenous saline infusions, was 3.2 %, whereas 6 4% of the controls, treated with saline infusions only succumbed

(*b*) The sulfaguanidine-treated patients passed fewer stools per day than the controls and required lesser amounts of saline

In contrast to these favourable experiences, Carruthers (1942) maintained that sulfaguanidine was of no value for the treatment of cholera. He noted in this connexion that there were seven deaths among 50 patients given this drug as well as hypertonic saline and sodium carbonate *per os*, whereas 15 out of the 88 controls, treated with infusions and alkali only succumbed. The difference in the fatality rates (14% as against 17%) was statistically insignificant. However, favourable reports on the therapeutic efficacy of sulfaguanidine were rendered by the following subsequent workers

Working in Kweilin China, Huang (1944) observed only one death among 22 cholera patients treated exclusively with sulfaguanidine and the necessary cardiac stimulants Similarly Misra (1944) had only one fatality among 16 patients given 5 g of the drug on admission 2 g every six hours until there were no more than five stools per day and then 1 g every eight hours until recovery—apparently in combination with the necessary infusion treatment The mortality in 70 controls was 31 4%.

Lahiri (1945) observed a mortality of 14 9% among 114 patients treated with sulfaguanidine as against fatality rates of 28.5% in 25 sufferers given sulfathiazole and of 29.5% in a calomel-treated control series of 176 patients

Further favourable results of sulfaguanidine treatment of patients hospitalized in Calcutta were recorded by Gupta and co-workers (1945) thus

	Clinically diagnosed cholera			*Bacteriologically confirmed cholera*		
Kind of treatment	*treated*	*died*	*mortality (%)*	*treated*	*died*	*mortality (%)*
Sulfaguanidine and saline infusions	263	3	1 1	158	3	1.9
Saline infusions only	262	13	4.96	157	11	7.0

Gupta and his colleagues added that

(*a*) The sulfaguanidine treated patients passed fewer stools per day than the controls and required lesser amounts of saline

(*b*) 83 7% of the sulfaguanidine-treated patients admitted in an anuric state passed urine within 24 hours, while the same was the case in only 31.9% of a corresponding control group

(*c*) None of the sulfaguanidine-treated patients developed uraemia, and seven patients showing pre uraemic symptoms recovered when given this therapy, which had hitherto not been used

An equally favourable report was rendered by Napier (1946) who stated that he had lost none of 60 sulfaguanidine-treated patients, whereas the mortality in a control series treated with saline infusions only amounted to 6%.

Referring to the treatment of cholera patients under field conditions, Seal (1946 1947) recorded only two deaths in a series of 134 sulfaguanidine treated sufferers while there were 67 deaths among 156 controls. The

sulfaguanidine dosage used was 3 g initially followed by 3 g every three hours until there were no more than two stools per day when 1 g of the drug was given at six hourly intervals for one more day As maintained by Seal (1946) only few of the patients, whose sulfaguanidine treatment had been started early required saline infusions

Similarly Pasricha and co-workers (1947b) reported a fatality rate of 18 3% in a series of 60 patients treated in their village homes with sulfaguanidine alone whereas 40 7% of 59 controls succumbed The corresponding figures in a group of 1118 patients treated in a Calcutta hospital with sulfaguanidine in combination with the usual infusion treatment and in a control group of 1170 sufferers receiving the latter only were 3 7% and 7.5%

Comparing the efficacy of treatment with sulfaguanidine and also with sulfadiazine with that of calomel treatment, Lahiri (1948) found that much better results were obtained if administration of these drugs was commenced before the patients had become collapsed than after the radial pulse of the sufferers had become imperceptible The corresponding figures were as follows

Method of treatment	*With radial pulse on admission* treated	died	mortality (%)	*Without radial pulse on admission* treated	died	mortality (%)
Sulfaguanidine	62	4	6.4	49	10	20 4
Sulfadiazine	71	6	8.4	68	17	24 7
Calomel (controls)	27	3	11 1	16	5	31.2

In contrast to these favourable results, Chu and co-workers (1946) recorded one death in a series of 25 cholera patients treated with sulfaguanidine as well as with saline infusions and one death in 29 controls receiving only the latter

El Ramli (1948) also found no difference in the mortality rate of 179 cholera patients treated with sulfonamides (mostly with sulfaguanidine in a minimal dosage of 12 g daily for 5 days) and of a control group of 182 sufferers, the fatality rates amounting respectively to 6 7% and 6 6%

The altogether unfavourable opinion held by El-Ramli regarding the value of sulfonamide treatment in cholera was fully endorsed in a further publication by Lahiri (1951) who recorded in this connexion that

"The effects of sulphaguanidine, formosulphathiazole and formosulphacetimide therapy and also of control with no chemotherapeutic drugs were studied in this series in 72, 64 61 and 71 patients respectively the respective case mortality rates being 30.55%, 34.37%, 34 43% and 18.31%. In severe cases admitted with imperceptible radial pulse death rates were 45 16%, 48 75%, 43.33% and 32.26% respectively respective numbers of cases being 31 41 30 and 31 In the study of age groups the lowest mortality rates were also obtained in the control group This was true for vibrio-positive and vibrio-negative patients."

Lahiri emphasized that the sulfonamides used by him evidently did not exert any appreciable vibriocidal effect *in vivo* the causative organisms disappearing from the stools of the sulfonamide-treated patients and the

controls at practically the same rate Further establishing through estimation of their concentration in the blood that all the sulfonamides tested by him were absorbable Lahiri maintained that

> "A sulphonamide may be an additional burden on the already dysfunctioning excretory system, especially if associated with irritant compounds like formaldehyde as may be seen in increased mortality figures for uraemia in persons receiving the latter drug."

Referring to 1908 cholera patients who received sulfaguanidine in addition to the necessary intravenous saline treatment Chakravarty (1954) stated that the high death rate (29 6%) observed in this group proved that sulfaguanidine was not a suitable therapeutic agent for such sufferers.

Other sulfonamides

Results obtained in the treatment of cholera with sulfonamides other than sulfaguanidine are summarized in the following tabulation

Drug	*Findings*
Sulfapyridine	Considered not advantageous by Misra (1944)
Sulfathiazole	Found unsatisfactory by Lahiri (1945) (see above)
Sulfadiazine	According to the 1945 report of the Indian Research Fund Association there were 2 deaths among 144 patients treated with sulfadiazine as against 9 deaths in an equally large control group. Favourable, but statistically insignificant results were recorded by some subsequent observers, e.g. by Chu & Huang (1946) Chu et al (1946) and Tao Woo & Loh (1948) Lahiri (1948) found sulfadiazine almost as useful for cholera treatment as sulfaguanidine. However Pasricha and co-workers (1947a) observing two groups totalling over 800 patients, reached the conclusion that sulfadiazine, administered in 1-g doses every 4 hours during the acute stage of the disease and 3 times a day for 48 additional hours "had no beneficial effect on cholera"
Succinylsulfathiazole	As quoted by Pasricha et al (1947d) according to a report of the Indian Research Fund Association (1944) succinylsulphathiazole had not given results superior to those in a control group treated only with saline infusions. Giving this drug in a dosage of 3 g 4-hourly during the acute stage of cholera and twice daily for two more days to 195 patients also treated with saline infusions, Pasricha and co-workers noted a mortality of 5 6 %, while that in a control group treated with saline infusions only was but insignificantly higher (6.2 %)
Formosulfathiazole	Bhatnagar et al (1948) stated that, using formosulfathiazole as the sole remedy for treatment of cholera patients in their rural homes, they had been able to save all but 3 of a series of 85 sufferers and

Drug	*Findings*
Formosulfathiazole *(continued)*	that none of the 28 of these patients whose treatment had been started before the onset of anuria had succumbed. Similarly Abdulla & Rohim (1950) recorded only 3 deaths among 43 patients apparently treated in a village with the drug under review even though fluids could be given by the oral route only. However, as noted before, Lahiri found formosulfathiazole unsatisfactory for the treatment of cholera.
Phthalylsulfathiazole	Pasricha et al. (1947c), using this drug for the treatment of 331 cholera patients, recorded a mortality of 7.3%, which was thus not significantly lower than that of 10.1% in 335 controls treated with saline infusions only. Narayana and co-authors (1954) noted a mortality of 5% in 40 patients treated with the drug as against a fatality rate of 12.5% in 57 controls.
Phthalylsulfacetimide	Observing only one death among 40 patients treated during the 1947 Egyptian outbreak with phthalylsulfacetimide, Seneca & Henderson (1949a) were favourably impressed by this drug. However, in a subsequent publication (1949b) they stated that its therapeutic value was lost if it was given later than the third day of illness, i.e. at a time when the vibrios had usually disappeared from the intestinal tract.
Formosulfacetimide	As mentioned before, Lahiri (1951) reported unfavourably on the use of formosulfacetimide for the treatment of cholera.
Acetylphthalylbenzenesulfonamide	According to Konar and co-workers (1953) results of treatment of 46 cholera patients with this drug were not significantly different from those in a control group of 42 patients even as far as the amount of saline needed for rehydration was concerned.

As will be gathered from the observations recorded above the history of cholera treatment with sulfonamides is rather similar to that of bacteriophage therapy, the initial belief in the marked efficacy of the former as well as of the latter therapeutic method not having been strengthened but rather disproved by the findings of subsequent workers. As is to be expected under these circumstances, again as in the case of the bacteriophage therapy the initially optimistic attitude of the writers commenting on the value of the sulfonamide treatment of cholera has changed into one of scepticism or even of frank disbelief. Thus it is noteworthy that Lahiri, formerly an advocate of this therapeutic method, concluded his 1951 article by stating:

"Chemotherapeutic drugs, to be of any real value in cholera, must have a quicker action than any of the sulphonamides tested in this series, and also should not be toxic."

An editorial appearing in the issue of the *British Medical Journal* in which Lahiri's article was published endorsed the opinions of this observer by stating that

"By the time the patient shows any definite symptoms of cholera it is probably too late for the drug to have any real beneficial action there has been a mass multiplication of vibrios, and their destruction without coincident antitoxin production will temporarily increase the endotoxin in the intestinal tract."

In contrast to these statements, Henderson & Seneca (1951) expressed the belief that the sulfonamides were of some value for the treatment of cholera. While certain that only "unabsorbable" compounds should be used they were "in view of irregularities of its behavior" not in favour of sulfaguanidine but recommended first phthalylsulfacetimide and secondly, succinylsulfathiazole Unfortunately however, in view of what has been stated above one cannot share the belief in the efficacy of the latter drug, while not enough is known about the former to establish its value for the treatment of cholera Generally speaking, the present writer is disinclined to believe that the use of any sulfonamide is of real advantage in the case of cholera patients whose condition has become serious enough to necessitate the administration of saline infusions.

Whether sulfonamides are useful for cholera treatment before this stage of the disease has been reached is still uncertain

Antibiotics

Initial use of antibiotics for the treatment of cholera seems to have been made by Amberson (1945) from whose report it can be gathered that (*a*) in addition to "supportive" saline treatment, he gave penicillin, usually in a total dosage of 200 000 units for adults to 57 patients, and (*b*) to another group of 58 patients he gave sulfadiazine in addition to treatment with saline infusions and penicillin No separate statistics were given for these groups Amberson s total results with "chemotherapy" (including that with penicillin) as compared to those obtained by the additional use of plasma will be recorded in a later part of this chapter

An attempt to use streptomycin in addition to saline infusions for the treatment of cholera was made by Reimann and co-workers (1946) but observations on the 10 patients thus medicated showed that

"Other than prompt reduction of the numbers of vibrios in the stools and slight shortening of the attacks, there is no evidence that streptomycin given orally or parenterally influences the course of the disease. Strains of *V comma* vary greatly in their resistance to streptomycin in vitro"

Further noteworthy results obtained when using various antibiotics (1) for laboratory studies with *V cholerae* and (2) for the treatment of cholera patients may be summarized thus

Laboratory investigations

Gauld and co-workers (1949) established that chloramphenicol (*a*) produced complete inhibition of the growth of *V cholerae* if used in a concentra

tion of 0 005 mg per ml of culture medium, and a 50% growth inhibition in half that dosage and (*b*) was effective for the treatment of intraperitoneally cholera infected mice provided that treatment was started not later than two hours after infection. Control experiments showed that sulfadiazine was endowed with a lower chemotherapeutic activity

Since preliminary experiments (Felsenfeld et al 1950a, 1950b) had shown that the susceptibility of cholera vibrios to antibiotics increased during storage of the strains, Felsenfeld and co-workers (1951) used 53 strains recently isolated in various areas for *in vitro* tests with 10 antibiotics, including penicillin, streptomycin, chloramphenicol, chlortetracycline (Aureomycin), neomycin oxytetracycline (Terramycin) and bacitracin. It was found that, while the cholera strains from Bombay and from Egypt were all sensitive to the antibiotics, several strains from Bengal, Assam and Indochina were refractory to most of these drugs in doses smaller than 100 units or μg per ml of culture fluid However no organisms were encountered which were resistant to neomycin or oxytetracycline

Continuing their studies Felsenfeld & Soman (1952) found that oxytetracycline and neomycin proved effective *in vitro* against all but one out of 100 cholera strains in doses of less than 100 μg or units per ml of medium while streptomycin chloramphenicol, bacitracin and chlortetracycline, used in the same doses did not inhibit the growth of more than 20% of the strains. Penicillin and sulfadiazine failed to affect 35% and 39% respectively of the strains

To test these therapeutic substances further mice were intraperitoneally infected with mucin-suspended cholera vibrios and then given the various drugs in different dosages by the oral route on the first, second or third day after infection It was found that (*a*) antibiotics poorly absorbed from the intestine such as streptomycin, neomycin and bacitracin, were ineffective (*b*) chloramphenicol and chlortetracycline gave better results and (*c*) oxytetracycline in doses of less than 100 mg per kg of body weight per day *was effective in 98% of the tests.*

Monkeys (*Macaca rhesus*) which had been intragastrically infected with *V cholerae* were orally treated with various doses of the drugs under test, medication being commenced when the first symptoms appeared and being continued for three days It was established that those animals which had been treated with adequate doses of oxytetracycline (25 mg per kg of body-weight per day) or neomycin (50 000 units per kg per day) could be invariably saved These two antibiotics were also found efficacious if administered parenterally to orally cholera infected monkeys in doses corresponding to those of 600 mg of oxytetracycline or 300 000 units of neomycin per day for an adult man

Studying the action of oxytetracycline and chloramphenicol on mice intraperitoneally infected with mucin suspensions of *V cholerae* Olejnik & Davidovitch (1951) found that if *oxytetracycline* in a dosage of 5-10 mg/kg

was administered intraperitoneally half an hour after infection all mice inoculated with Inaba strains of *V cholerae* recovered in the case of an Ogawa strain a dosage of 15 mg/kg was required to obtain the same result

Larger doses of the antibiotic were necessary to obtain 100% survival of mice infected five hours before treatment—15 mg/kg in the case of one Inaba strain 30 mg/kg for the other two Inaba and the Ogawa strains

If oxytetracycline was administered to the animals later than five hours after infection even heroic doses were ineffective

As to *chloramphenicol* Olejnik & Davidovitch found that this drug given half an hour after infection was curative in doses of 25 mg/kg in the case of one Inaba strain of 50 mg/kg in the case of the two other Inaba strains, and of 100 mg/kg in the case of the Ogawa strain used

Five hours after infection 250 mg/kg of the drug still proved effective in the case of two of the Inaba strains but this dose was but partially effective (30% survivals) in the case of the third Inaba and the Ogawa strains

Further *in vitro* tests with oxytetracycline were made by Das and co-workers (1951) and by De and colleagues (1952) The former observers found that the antibiotic completely inhibited the growth of *V cholerae* at a concentration of 2 5 μg per ml of medium As summarized in the *Tropical Diseases Bulletin* (1953) De and co-workers recorded that oxy tetracycline (Terramycin)

"was bacteriostatic at pH 7 4 in a concentration of 1 μgm./ml. while chloramphenicol required 1 or 10μgm /ml. at higher pH up to 10 or 100 μgm./ml. of terramycin was required to produce bacteriostasis, but only 0.1 10 μgm./ml of chloramphenicol was required for this purpose. For bactericidal action, larger concentrations were required, but while the action of terramycin was less marked in alkaline medium, that of chloramphenicol remained the same or improved with a rise of pH"

It will be noted that as far as these tests went chloramphenicol gave more satisfactory results than oxytetracycline

Working with chlortetracycline Seal and co-workers (1951) found this antibiotic vibriostatic in a 15μg/ml dosage and vibriocidal in a dosage of 50μg per ml of medium.

According to a review in the *Tropical Diseases Bulletin* (1954) Nagao (1953) testing various antibiotics and homosulfanilamide in the laboratory found chloramphenicol and roseomycin (isolated from *Streptomyces roseus*) more effective in *in vitro* tests as well as in experiments with intra peritoneally cholera infected mice than the sulfonamide and streptomycin.

Studying the sensitivity of *V cholerae* to antibiotics with the aid of *in vitro* tests and experiments with orally infected guinea pigs, Gohar (1953) found that

(*a*) Dihydrostreptomycin chloramphenicol, chlortetracycline and oxy tetracycline exerted a more potent bacteriostatic action than sulfonamides like Sulfasuxidine (succinylsulfathiazole) sulfaguanidine and sulfadiazine

(*b*) Among the above-mentioned antibiotics, dihydrostreptomycin alone exerted an appreciable bactericidal action on *V cholerae*

(*c*) The cholera vibrios readily acquired a resistance to dihydrostreptomycin, less readily to chloramphenicol, and least to the other two antibiotics.

(*d*) As far as the *in vivo* tests were concerned chloramphenicol and dihydrostreptomycin, used alone or in combination with sulfonamides, gave comparatively the best results

The conclusion reached by Gohar was that

"Because of its bactericidal action and lack of absorption from the intestines, dihydrostreptomycin, either alone or in combination with sulphaguanidine or sulphasuxidine, is recommended for trial in the treatment of cholera."

Experimenting with chloramphenicol, chlortetracycline hydrochloride (Aureomycin) and oxytetracycline hydrochloride (Terramycin) Dutta & Habbu (1954) established that these antibiotics were active *in vitro* against large as well as small inocula of *V cholerae* If administered in 100 mg/kg doses orally 8 hours after intra intestinal cholera infection of young rabbits, the three antibiotics prevented the appearance of symptoms. The same result was still obtained when treatment was commenced 16 hours after infection but the antibiotics failed to have any effect when given 24 hours after infection, *i.e* after diarrhoea had started. Results were not improved when treatment with chlortetracycline commenced at that time was combined with saline administration

Testing *in vitro* the sensitivity of 166 strains of the Inaba type of *V cholerae* to crystalline penicillin G dihydrostreptomycin tetracycline chlortetracycline and chloramphenicol D C. Lahiri et al (1956) noted that each of these antibiotics

"showed a very wide range of concentrations between which the most sensitive strains and the least sensitive strains showed their susceptibility The concentration of a given antibiotic at which all strains were completely inhibited showed itself to be even the 50 to 100fold of that at which only the most sensitive strains were completely inhibited"

Nevertheless it could be established that tetracycline chlortetracycline and chloramphenicol exerted generally speaking, a more marked inhibitory action than the other antibiotics enumerated above.

Treatment with antibiotics

Attempts to treat cholera patients with *chloramphenicol* were first made by Chaudhuri and co-workers (1950) who administered the drug orally in a total dosage of 6 g on the first day after admission and in total dosages of 3 g each on the second and third days to 10 patients. The necessary infusion treatment was given at the same time to these sufferers who admitted on the average nine hours after onset of the disease, were still in the evacuation

stage or in the collapse stage There was no obvious difference in the clinical appearances shown by these patients and by an analogous control group two of the treated and one of the controls died It was found however that the number of cholera colonies developing from the stools of the antibiotic-treated patients was markedly reduced after 24 hours and that there was no growth of *V cholerae* from the stools collected after 48 hours, whereas the organisms persisted in the faeces of the controls for periods up to seven days

In the course of a further investigation Chaudhuri and colleagues (1952) compared the efficacy of oral and intravenous treatment with chloram phenicol with that of saline treatment alone observing in each case a group of 20 patients who had been ill with cholera for 1 20 hours.

The total dosage of the antibiotic given orally to adults was at first 9 g, but was afterwards reduced to half 0 5 g being given on admission, then 0.25 g every hour for six hours and finally 0.5 g every six hours until the total of 4 g had been administered For intravenous use dilutions of the drug were made in 10-12 ounces of saline solutions. An initial dose of the antibiotic amounting to 0 5 g was followed by 0.25-g doses at six hourly intervals until a total of 1 g-1.25 g had been reached

There was no death among those intravenously treated with chloram phenicol as against four deaths in the orally treated and three fatalities in the controls. While admitting that these differences were not statistically significant, Chaudhuri and co-workers stressed that (*a*) diarrhoea termi-nated earlier in those treated with the antibiotic, and (*b*) except in one instance the vibrios disappeared much earlier from the stools of the treated than from those of the controls

Chakravarti et al. (1954) used chloramphenicol for the intravenous treatment of 50 cholera patients, most of whom had been ill for 6-20 hours. The earlier of these sufferers received an initial dose of 0 5 g followed at six-hourly intervals by 0.25-g doses, but to a later group of 38 patients apparently three injections of 0 5 g were given at four hourly intervals Again there were no clinical differences between the antibiotic-treated sufferers and an analogous control group five of the former and seven of the latter died It was noted, however that (*a*) the stools of the patients receiving chloramphenicol became more rapidly formed than those of the controls and (*b*) *V cholerae* disappeared from the stools of most of the former by the third day while half of the controls continued to excrete the organisms for periods ranging from four to seven days.

In the course of an investigation, to which reference has been made above Narayana and colleagues (1954) administered chloramphenicol in a total adult dosage of 9 g, dispensed in 0.5-g doses at four hourly intervals to 57 cholera patients who had been ill for 2 14 hours on admission. An analogous control group received two keratin-coated pills containing 2 grains of potassium permanganate each every four hours up to a total

of 36 pills Both the antibiotic treated patients and the controls also got the necessary amounts of hypertonic glucose-saline solution There were seven deaths among the controls as against four deaths of patients treated with chloramphenicol *V cholerae* was found to be present in the stools of the latter for an average of 2.8 days as against 3 8 days in the controls.

Treatment of cholera patients with *oxytetracycline* was commenced in 1951 by Konar & Sengupta as well as by Das and colleagues The first mentioned two workers administered the drug orally in 500-mg doses every six hours for three days (average total dose 24 capsules of 250 mg each) to 50 cholera patients They were also given the necessary infusion treatment, while 50 controls received only the latter There were four deaths in the antibiotic treated group as against three in the controls. However the stools of the former group were found to be free from cholera vibrios after an average of 48 hours after admission while in the case of the controls the same result was obtained after more than three days In the opinion of Konar & Sengupta the success of oxytetracycline treatment depended upon administration of the drug quite early in the disease.

Das & co-workers (1951) orally administered oxytetracycline to 36 cholera patients in a dosage of 1 g on admission, then gave half this dose after two hours, and finally 0 5 g at six hourly intervals until the third day of treatment (total dosage 6-7 5 g) this medication was combined with the usual infusion treatment There were six deaths among those receiving the antibiotic as against eight in the 35 controls who were treated in the usual manner The causes of death in the two groups were

	Antibiotic treated	*Controls*
Peripheral circulatory failure	2	5
Hyperpyrexia	1	2
Uraemia	3	1
Totals	6	8

The stools of the patients treated with oxytetracycline became free from cholera vibrios markedly sooner than those of the controls.

In a further paper Das et al. (1953) reported upon the results of oral treatment with this antibiotic in 190 cholera patients as compared with those in 82 controls receiving only the saline infusions also given to those medicated with oxytetracycline The fatality rates in the two groups were

Time of commencement of treatment	*Antibiotic treated*	*Controls*
Within 6 hours of onset	4.3 ⁄	8.5 ⁄
6-12 hours of onset	20.3 ⁄	30 0 ⁄
Totals	11 6 ⁄	18.3 ⁄

Considering these differences to be statistically insignificant, Das and colleagues expressed the opinion that oral treatment with oxytetracycline was of no additional advantage in the management of cholera patients.

Das Ghosal & Gupta (1953) likewise observed no significant differences in the case mortality when comparing the therapeutic results in 20 cholera patients treated intravenously with oxytetracycline with those in 16 controls

Giving oral doses of this antibiotic to seven cholera patients also receiving infusions, De & co-workers (1954) noted that its administration seemed to prevent the rise of the serum bilirubin level to the peak value observed in controls on the second day of illness Commenting upon these findings as well as upon the therapeutic results of the above-quoted workers De and colleagues stated that

"The clinical reports cited above hold out very little promise of success for oxytetracycline therapy in cholera, although the vibrios in the stool disappear rather earlier Nevertheless the present study of a small number of cases suggests this antibiotic has some definite beneficial effect on at least one perhaps ominous, pathological process going on inside the body in cholera. Perhaps higher doses given more frequently especially at the onset of illness would have more obvious clinical results"

A preliminary attempt to treat cholera patients with *chlortetracycline* was made by Seal Ghosal & Ghosh (1951) who for this purpose at first resorted to intravenous administration of three 100-mg doses of the antibiotic at six hourly intervals but afterwards used only half these doses Results of this treatment which was combined with saline infusions were not satisfactory since there were four fatalities among the 12 patients concerned whereas only three of the controls succumbed. However Seal and his colleagues pleaded that by chance the antibiotic treated patients had been more seriously affected than the controls and maintained that both the duration of illness and that of vibrio excretion appeared to have been reduced by one third in those receiving chlortetracycline One cannot help noting, however that while uraemia became manifest in only four of the controls, it developed six or seven times in those treated with the antibiotic It also deserves attention that the initial administration of the original dose (100 mg) led to a febrile reaction

Results of a further trial in treating cholera patients with chlortetracycline by Seal and co-workers (1954) were as follows

Method of treatment	*Treated*	*Death*	*Mortality (%)*
Chlortetracycline	50	7	14 0
Sulfaguanidine	35	5	14.3
Infusions only	35	5	14 3

Note The necessary infusions were also given to the patients of the first two groups. Chlortetracycline was administered orally in doses ranging—according to the severity of the attacks—from 6.65 g to 4 875 g.

Seal and his colleagues stated that administration of this antibiotic shortened the acute stage of the disease the duration of anuria and the period of vibrio excretion. It is noteworthy however that uraemia developed five times in the patients treated with chlortetracycline The incidence

of uraemia was also high among the sufferers receiving sulfaguanidine (four instances) while this complication was noted but once in the control group Jaundice was observed in two chlortetracycline-treated sufferers and in one given sulfaguanidine.

Narayana and co-workers (1954) orally administered chlortetracycline to 40 cholera patients also given the necessary infusion treatment. Adults received two capsules of 0.25 g each at six-hourly intervals, while those below 20 years got alternately two capsules and one capsule at the same time intervals, the total dosages apparently amounting respectively to 24 and to 18 capsules Only one of the patients of this series died. Narayana and colleagues added that chlortetracycline

"produced an appreciable clinical improvement within a period of 12 to 24 hours of starting the treatment in most cases. A sense of an all-round well-being, with the pulse regaining more or less in volume and tension, a marked decrease of dehydration, the state of collapse disappearing almost completely and above all, the Rice-water stools giving place to formed motions"

They also noted that the stools of the patients treated with this antibiotic became negative for *V cholerae* at an average of 1.9 days as against 2.8 days in those treated with chloramphenicol In their opinion chlortetracycline

"holds out the promise of a drug worthwhile trying in the treatment of cholera in conjunction with the time-honoured intra-venous saline"

However even if one could agree with this contention,[1] one must stress that the aim of a specific cholera therapy is not to augment the value of the infusion treatment but to render the latter superfluous by suppressing the morbid process before dehydration and collapse have developed. Thus far no convincing evidence has been obtained that any of the methods of treatment discussed above falls into this category of a *therapia sterilisans magna*[2] Moreover while one must hope that such an effective method of suppressive treatment will eventually be evolved, one must realize that in actual practice its usefulness would be limited, since, owing to the rapid progress of the disease, many of the cholera patients are already in a state of dehydration and collapse when first seen. Thus, whatever the future may hold, it is difficult to visualize a time when the infusion treatment will not occupy a prominent place in the therapy of cholera.

Since thus far no satisfactory specific method of cholera treatment has been evolved, the question how to deal with patients seen in the earliest

[1] Further results obtained with chlortetracycline in the case of 21 cholera patients by Leigret (1955) were frankly disappointing, no fewer than 12 of the sufferers succumbing.

[2] The same holds true of the treatment of cholera patients with the juice of an Indian plant, *Calamus aromaticus*, recommended by Chatterjee (1953a, 1953b). For even though this drug was used in combination with a proprietary medicine to stop the vomiting of the patients (see below), this combined therapy obviated the need for parenteral saline administration in but 17% of 1093 cholera patients, proving successful in the case of 35 individuals with mild attacks and in 133 persons suffering from the disease in a moderately severe form.

stage of the disease remains unsettled In an interesting discussion of this problem Strong (1944) declared

"Long experience with the use of castor oil neutral salts and other purgatives, including calomel, has demonstrated that treatment with these drugs frequently if not usually exercises an unfavourable influence over the course of the disease In the human intestine the cholera organism multiplies most rapidly in a fluid medium moreover the action of these purgatives tends to increase the catarrhal condition and to impair the resisting power of the mucous membrane of the intestine. Therefore, the purgative treatment during this stage cannot be recommended and the indications are to limit peristalsis and to put the intestine at rest."

Accordingly Strong recommended strict rest in bed for the patients seen in the earliest stage of cholera. Unnecessary bathing, changing of bed linen and the like had to be avoided so as to move the patients as little as possible. No food except rice water or barley water was to be allowed In his opinion administration of $\frac{1}{4}$ grain doses of morphine with $\frac{1}{150}$ grain of atropine hypodermically or of 15 minims of chlorodyne orally were useful during the first day of illness but these drugs had to be avoided after 24 hours from the onset

While it is a debatable point whether opiates or opium derivatives ought to be given to cholera patients even quite early in the disease, Strong s advice to avoid drastic medication or other exhausting therapeutic proce dures at this stage deserves full consideration Whatever method of treatment is chosen a careful watch over the patients must be instituted so that dehydration and collapse may be counteracted by suitable means as soon as they begin to be manifest. As said above, cholera patients often unfor tunately reach the hospitals in a condition necessitating rehydration by parenteral routes. Thus the question how to deal therapeutically with the earliest stage of the disease is quite often rather of academic interest than of great practical importance

Infusion Treatment

Introductory remarks

Though Hermann (1831) tried during the 1830 Moscow outbreak to cure cholera patients by the injection of water into their veins, by common consent Latta is accorded the credit of having initiated the method of infusion treatment of which ample and most beneficial use is still being made As Latta stated in his original publication a report reproduced in the *Lancet* (1831 32) he was motivated to consider this therapeutic method on account of O'Shaughnessy s observations (1831 32) on the ominous loss of fluids and salts from the blood of cholera patients His attempts to correct this deficiency through rehydration by the oral or rectal route having

failed, he "at length *resolved to* throw the fluid *immediately into the* circulation" (i.e. into the basilic vein) Latta dissolved for this purpose

"from two to three drachms of muriate of soda and two scruples of the subcarbonate of soda in six pints of water and injected it at a temperature of 112°F"

Unfortunately the first patient treated according to this new method though showing signs of an almost miraculous improvement of her collapsed condition after infusion of six pints or about 3 5 litres succumbed because Latta was not recalled when a relapse took place However another sufferer to whom under these circumstances a second infusion was administered, became cured In two further papers (1832 33a, 1832 33b) Latta referred to five patients treated according to his method of whom three recovered As narrated by Greig (1946) in an interesting account of Latta s achievements, ampler use was made of the method of this worker during the 1832 cholera epidemic at Edinburgh by Mackintosh (1836) who employed the following solution

Sodium chloride	½ ounce (later 1 ounce)
Sodium bicarbonate	30 grains (later 60 grains)
Water	10 pounds (pints)

Note Addition of egg-albumin was tried but found to be of no beneficial effect.

Though only 25 of the 156 patients treated with this fluid recovered, Mackintosh felt convinced of the value of intravenous infusions, stating that

"Should I ever have charge of cholera patients again, I shall, profiting by the experience I now possess, use the saline solution at an earlier period of the stage of collapse nay at its commencement, in order to lessen the thickness of the blood before organic mischief is done."

However other workers were more or less sceptical, Griesinger (1857) for instance, maintaining that the experiences with the intravenous infusion therapy of cholera

"have been so unfavourable thus far that it cannot at all be recommended, even though it is true that, as indicated by the often marked and sudden improvement of the patients immediately after the injection the method is not wrong, and one must admit that so far the overwhelming majority of the injections has been administered to patients in a hopeless condition [Trans.]

In view of the fact that intravenous administration of fluids was not rarely supposed to be harmful it is not surprising to find that attempts have been made to rehydrate cholera patients by other routes even with the aid of baths, inhalation of water vapours or by introduction of saline solutions into the urinary bladder (Piorry 1849 quoted by Mettenheimer 1892) While, naturally such weird procedures were but passingly used, the introduction of the method of subcutaneous saline administration to cholera patients, which Cantani (1892) claimed to have first proposed in 1865 attracted much attention However while in the nineteenth century

particularly this procedure has been often used in place of intravenous infusions more recently the latter method once more became the standard for the treatment of cholera

It was the great merit of Rogers (see Rogers & Mackelvie 1908 and Rogers 1909a) to have drawn universal attention to the use of hypertonic saline solutions in place of the normal or often even more or less hypotonic fluids used by the earlier cholera workers It is however historically interesting to note that as early as 1893 Gaertner & Beck as a result of experimental observations on dogs recommended "over-salting" the blood of cholera patients through the intravenous administration of concentrated saline solutions Acting on this advice Rosner (1895) used a 10% NaCl solution in quantities of 300 ml for the treatment of seven cholera sufferers but *in spite of an invariable temporary improvement lost* four of them Evidently Rogers was unaware of this early and rather discouraging result

Though some of the earliest observers like Lewis (1832 33) had already drawn attention to the advisability of giving alkalis to cholera sufferers by the oral route and other early workers had resorted to infusions with weakly alkaline salt solutions it is to the great credit of Sellards (1910) that he pioneered the rational use of adequately alkaline fluids in the treatment of the disease Soon afterwards several workers—first Kausch (1911 1916) and then Whyte (1913 1915) Gaertner (1915) and Strauss (1915)—advocated the use of glucose solutions for the treatment of cholera.

As will be gathered from the following disquisition the method of administering alkaline solutions to cholera patients which was again recommended by Rogers (1915 1916b) has been almost universally adopted. The question whether preference ought to be given to hypertonic or normal saline solutions, on the contrary is still much debated

Preparation of infusion fluids

Regardless of the kind and the concentration of the fluids employed for the infusion treatment of cholera, it is of the utmost importance to use suitable water for their manufacture It was known for a long time that however efficacious such fluids were their administration often led to untoward reactions consisting of chill fever and sometimes even collapse As aptly stated by Pasricha, Malik & Paul (1941) these reactions were

"explained on various obsolete complex and unsubstantiated theories involving the doctrine of specific ion effect, haemolysis, hydrogen-ion concentration, etc. or more often simply regarded as inevitable after-effects of intravenous therapy"

In contradiction to these and other unsatisfactory postulations, work initiated by Hort & Penfold (1911) and continued by other observers, the next being Seibert (1923) but long ignored by the cholera workers has furnished evidence of the presence of fever producing or as they are usually

called, pyrogenic substances in the infusion fluids causing untoward reactions. It was established that these pyrogens were filterable thermostable products of the growth of certain bacterial species which, being ubiquitous, could easily infest any water not kept under absolutely sterile conditions. Even at room temperature formation of the pyrogens was apt to take place within one or at most a few days, and, since these substances were not destroyed by ordinary autoclaving and were capable of passing over with the steam unless redistillation of pyrogen-containing waters was carried out under special precautions, the pyrogens failed to be destroyed in the course of the procedures ordinarily used for the preparation of infusion fluids. A further difficulty is that no fully reliable rapid method for the detection of the pyrogens is available, animal experiments forming the only means of ascertaining their presence or absence (see Bose & Ahuja, 1944).

While in the opinion of many observers the pyrogens alone are responsible for the untoward reactions produced by infusion fluids, some workers, e.g. more recently Paul & Chatterjee (1944) maintained that such reactions could also take place when the fluids injected were so alkaline or acid as to render the blood incapable of exerting a neutralizing or buffering action.

Before dealing with the methods actually used for the production of pyrogen-free fluids for the purposes of cholera treatment, attention has to be drawn to the statement of Lees & Levvy (1940) that in emergencies such fluids could be obtained by (*a*) adding powdered charcoal to the waters to be treated at the ratio of 1 g per litre (*b*) shaking for 15 minutes and (*c*) separating off the charcoal by filtration through paper

Thomas & Ting (1938) apparently the first to use pyrogen free solutions for the infusion treatment of cholera patients, resorted to the distillation of surface (canal) water in a special apparatus and sterilized the saline solutions made from the distillates within three hours after manufacture. The flasks in which the infusion fluids were kept were closed with plugs consisting of a gauze core covered with a layer of silk tissue. Apparently the infusion fluids thus prepared remained suitable for curative purposes for two weeks.

An easily applicable method of obtaining pyrogen-free water recommended for cholera work by Panja and colleagues (1942) was thus described in the 1943 report of the Indian Research Fund Association

> 2000 ml of tap water or of distilled water known to contain pyrogen were acidulated with 0.4 ml of strong commercial sulfuric acid and then treated with 4 ml of a 0.4 / solution of potassium permanganate. The fluid was then boiled for two hours and if during this process the colour disappeared, further additions of potassium permanganate were made to maintain at least a trace of colour
>
> The fluid thus prepared could be kept for a fairly long period, because pyrogen-producing organisms were incapable of developing on account of the acid reaction. However if accidental contamination with organic matters led to a decolouration of the fluid, it was safer to add more potassium permanganate and to boil again.

If needed for the manufacture of infusion solutions, the fluid was filtered through paper and, after 5-10 drops of hydrogen peroxide had been added was shaken When after 1 2 minutes the permanganate colour had disappeared the fluid was slightly heated so as to remove the excess of H_2O_2 After cooling the pH was adjusted to 7 1

A more refined method for producing pyrogen free water was outlined by Napier (1946) thus

"In a clean glass still, re-distil some freshly distilled water to which a little sulphuric acid and one or two crystals of potassium permanganate have been added to give a faint pink colour If during the process of distillation the pink colour disappears from the water in the still, a little more sulphuric acid and potassium permanganate must be added.

"The distillate is collected in a closed flask which has been previously prepared by rinsing first with a solution of potassium bichromate and sulphuric acid, then washed out, first with distilled water and then with pyrogen-free water and finally sterilized by autoclaving.

"The pyrogen-free water is sterilized in an autoclave and may be used for about 3 to 4 days."

A recent proposal by Schmidt & Blass (1955) was to utilize special membrane filters or EKS Seitz filters for the preparation of pyrogen free water However reliable and expedient though these up-to-date procedures certainly are it seems doubtful whether they would be widely applicable under the conditions usually prevailing in the cholera-affected areas

Sen Gupta (1945) recorded that running short of distilled water during a serious cholera outbreak he had made successful use of a hypertonic saline and glucose solution prepared with filtered and sterilized well water only four out of 95 patients succumbing Since the fluids used were not pyrogen-free shivering was observed after the infusions However in Sen Gupta s opinion this reaction had some beneficial effect as purging and vomiting were found to stop earlier in the patients receiving well water than in those treated with fluids prepared with distilled water

Commenting in an appendix upon these statements the editor of the *Indian Medical Gazette* expressed the opinion that

"For intravenous use, fresh pyrogen-free distilled water is of course highly desirable. In a cholera epidemic in rural areas, and often even in urban areas, fresh distilled pyrogen-free water is often unobtainable Old distilled water unless it has been stored in completely sealed and sterile containers, is often highly pyrogenic. Fresh undistilled water is often much better than doubtful old distilled water

"In the cholera wards in Calcutta hospitals, Calcutta tap water has usually been used, and while it gives rise to some febrile reaction, these are usually not very serious, and the results are usually good

"In rural areas, the editor has used fresh well water filtered and boiled, with good results. He always teaches In cholera give saline in the best water you have, but in case of necessity use well water tap water or tank water rather than leave a patient collapsed from cholera without a transfusion "

In agreement with this statement ample experiences of previous workers have shown that it is by no means a *sine qua non* to use distilled water in order to prepare infusion fluids for cholera treatment However care must

be taken to choose waters which are neither acid (as, in China at least, well waters not rarely are) nor show too high an alkalinity as surface waters not seldom do In spite of the assertions of Sen Gupta quoted above even under emergency conditions every possible effort must be made to use infusion fluids which produce no chills or other untoward reactions Should such reactions appear even if the infusion fluids are quickly used after preparation, then advantage ought to be taken of the method of pyrogen removal devised by Lees & Levvy which, though apparently not used thus far in cholera work, deserves serious attention on account of its simplicity and rapidity of action

In contrast to the practice formerly followed, some modern workers have with great reason recommended the use of various fluids for cholera therapy in order to adapt the infusion treatment to the varying conditions met with in the course of the disease Important formulas for these are tabulated below

(a) Recommended by Rogers (1921 1952)

1 Hypertonic saline solution

Sodium chloride	120 grains (8 g)
Calcium chloride	4 grains (0.25 g)
Water	1 pint (568 ml)

2 Alkaline saline solution

Sodium bicarbonate	100 grains (10 7 g)
Sodium chloride	90 grains (6.0 g)
Water	1 pint (568 ml)

(b) Recommended by Napier (1946)

1 Hypertonic saline solution

Sodium chloride	140 grains—16 g
Pyrogen free distilled water	1 pint—1 l

2 Alkaline saline solution

Sodium chloride	80 grains— 9 g
Sodium bicarbonate	180 grains—20.5 g
Pyrogen-free distilled water	1 pint—1 l

3 Alkaline hypotonic saline solution

Sodium chloride	60 grains—6.8 g
Sodium bicarbonate	180 grains—20.5 g
Pyrogen-free distilled water	1 pint—1 l

4 Bicarbonate solution

Sodium bicarbonate	440 grains—50 g
Pyrogen-free distilled water	1 pint—1 l

(c) Recommended by Shattuck (1951)

1 Isotonic saline solution

Sodium chloride	(90 grains)—9 g *
Water	(1 pint)— 1 l

* 8.5 g are usually recommended.

2. Hypertonic saline solution

Sodium chloride	14 g
Freshly distilled water	1100 ml

Reduce volume by boiling to 1000 ml.

3 Alkaline saline solution

Sodium bicarbonate	18 g
Sodium chloride	6 g
Freshly distilled water	1100 ml

Before addition of sodium bicarbonate, reduce volume by boiling to 1000 ml.

4 Dextrose (glucose) saline solution

Dissolve 50 g of the sugar in 1 litre of isotonic or hypertonic saline. It is desirable but not essential to add 2 mg of thiamine chloride.

A special technique has to be used for the preparation of the alkaline solutions enumerated above, because under the action of heat sodium bicarbonate becomes decomposed into sodium carbonate Hence as recommended by Rogers (1921) it is necessary to wrap the needed doses of sodium bicarbonate in paper and to sterilize the packets thus obtained by dry heat Then after adequate saline solutions have been made and sterilized the required amounts of sodium bicarbonate are added As pointed out by Rogers this addition renders the fluids slightly hazy but they remain quite safe for intravenous infusion, however these alkaline solutions should never be used for subcutaneous injections As pointed out by Chaudhuri (1950) the sodium bicarbonate doses might be enclosed in glass ampoules instead of in paper packages However the latter have been found to serve their purpose fully

An alternate procedure recommended in the 1945 US Army regulations for cholera treatment quoted by Shattuck (1951) was to add immediately after 0 6% saline solutions had been made up and sterilized by boiling, to each litre of these solutions 18 g of sodium bicarbonate taken directly from an original container and weighed in a sterile vessel The fluids thus prepared had to be used as soon as they had been cooled to body temperature

In order to avoid the minor difficulties attendant upon the preparation of bicarbonate-containing infusion fluids Banerjee & Datta (1936) advised replacing this chemical by sodium lactate because the solutions made with the latter could be sterilized by boiling and could be injected subcutaneously as well as intravenously However Chaudhuri (1950) though again recommending the use of M/6 solutions of sodium lactate for cholera treatment, admitted that these were efficacious only in the case of mild or moderately severe cholera attacks, while severely affected patients had to be treated with alkaline solutions prepared in the usual manner with sodium bicarbonate Most workers make exclusive use of the latter

Routes and technique of infusion

Before dealing with the routes and the technique of the infusion treatment it is necessary to assess the value of two simpler methods of restoring the fluid balance of cholera patients namely oral and rectal administration of fluids Dealing with the former of these two procedures, Rogers (1921) justly stated that, when treating patients affected with cholera gravis

"it is useless to give large quantities at once as they will only induce vomiting, but by allowing an ounce or two at a time with short intervals it is surprising how much may be retained and absorbed, greatly to the benefit of the patient. As long as the rectal temperature is not subnormal ice may be given to suck and may allay the irritability of the stomach. Theoretically it would appear to be sound to add a little sodium chloride to the water but practically the apparent advantage may be more than counteracted by limiting the amount drunk on account of the salt taste If for any reason transfusion

cannot be carried out, however I consider such an addition to be worthy of trial in severe cases in which the acuteness of the thirst will ensure its ready acceptance."

The administration of fluids *per os* may be suitably combined with that of alkalis but, as pointed out by Rogers & Shorten (1915) this often routinely practised medication cannot be relied upon to combat acidosis.

Ample use has been made in the past of rectal fluid administration to cholera patients, the more so as the fluids were often used as vehicles for drugs, especially astringents. In particular the method of tannin enteroclysis devised by Cantani (1884) was much utilized but, though it was still considered to be usually effective by Liebermeister (1896) it was afterwards given no more attention.

Rogers (1921) considered rectal fluid administration to be indicated in comparatively mild cholera attacks, when the blood pressure had not fallen below 70 mm, because it had been found that

"the large bowel retains its powers of absorption as long as there is a fair pulse, and the patient may often be tided over the danger of collapse by frequently repeated copious saline enemata"

He recommended that ½ 1 pint be given *per rectum* every two hours during the evacuation stage but that after the urine secretion had become satisfactory in the stage of reaction, the same amounts should be given at four hourly intervals. The solutions had to be injected slowly and as high up as possible with the aid of a long soft tube, and the patients had to be urged to retain the enemata as long as they could In the case of patients with a blood pressure not much over 70 mm, particularly in the case of children or elderly people, the rectal injections were preferably to be administered by a continuous method the rate of flow being regulated at from ½ to 1 ounce per minute Isotonic saline (90 grains of NaCl per pint) had to be used, to which, as long as the urine was acid, 100 grains of sodium bicarbonate were added per pint. Addition of 4 grains of calcium chloride per pint was advantageous. The rectally treated patients had to be closely watched so that, if necessary prompt additional use could be made of intravenous saline administration

Rectal fluid administrations were again recommended for the treatment of cholera by Rogers (1952) as well as by Strong (1944) and Napier (1946) Strong stressed in this connexion that intravenous infusions ought to be resorted to only when they were strictly indicated and that, even when they became necessary combined use ought to be made of intrarectal fluid administrations.

While admitting that, as long as the radial pulse can be felt, fluids injected into the peritoneal cavity of cholera patients are rapidly absorbed with a resultant improvement in the clinical condition Rogers (1921) was not in favour of this route of fluid administration He stated that he had used this method only occasionally in children, in whom it was difficult to find suitable veins for infusion, but that he had given it up afterwards,

because it was found that the mortality of cholera patients receiving fluids by this route was considerably higher than that among the intravenously infused sufferers Subsequent observers (see for instance Ghanem & Mikhail, 1949) also advised against cholera treatment with intraperitoneal infusions so that this method may be said to be of historical interest rather than of present importance However Napier (1946) still referred to the possibility of using it in the case of children or adults in whom no suitable veins could be found to make infusions He advised raising the foot of the patient s bed after intraperitoneal infusions had been made, because the absorptive powers of the pelvic peritoneum were poor Napier added, however that, if intravenous infusions were not possible, the subcutaneous or intramuscular route might be used in place of the intraperitoneal or the fluids might be administered into the marrow of the sternal bone or the tibia To carry out the last two methods under the often rather unpropitious conditions prevailing during cholera outbreaks would be rather difficult.

As has been stated in the introduction to the present disquisition during the latter part of the nineteenth century many workers resorted to subcutaneous infusions instead of continuing to use the original method of intravenous saline administration for the treatment of cholera. However though the efficacy as well as the supposedly greater safety of the former therapeutic method were often asserted some of the contemporaneous observers for instance, Rumpf (1892) and Rumpel (1894) expressed opposite opinions. Convincing evidence of the superior value of intravenous saline administration was furnished by Nichols & Andrews (1909) who recorded the following results in 450 cholera patients treated by different methods during the 1908 Manila outbreak

Method of treatment	*Patients*	*Deaths*	*Mortality (%)*
Stimulation	145	47	32.4 *
Subcutaneous infusion	175	117	66.8
Subcutaneous and intravenous infusions	36	19	52.7
Intravenous infusion	94	41	43 6

* The mortality in the first group treated with stimulants like strychnine and digitalin was lowest because it included the mildly affected patients. Isotonic saline solutions were used for the treatment of the other three groups.

Rogers (1921) while maintaining that the supposed simplicity of the method of subcutaneous infusion was apparent rather than real considered it much less efficacious than the intravenous method of saline administration Moreover the following grave objections had to be made to the infusion treatment by the subcutaneous route

(a) The difficulty of administering the large amounts of fluid (an average of 4 pints) required for the treatment of collapsed cholera patients

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(*a*) The difficulty of administering the large amounts of fluid (an average of 4 pints) required for the treatment of collapsed cholera patients

(*b*) The considerable pain caused by subcutaneous infusions, which aggravated the state of shock

(*c*) The slow absorption of the subcutaneously given fluids and the total absence of absorption in markedly collapsed patients

(*d*) The formation of abscesses at the site of injection in a certain number of the patients, due on the one hand to the low vitality of the tissues in cholera, and on the other hand to the difficulty of maintaining asepsis.

Nevertheless Rogers, though advising that the least possible use be made of subcutaneous infusions, maintained that

"If for any reason intravenous injections are impracticable frequently repeated subcutaneous salines should be used in all cases in which rectal injections fail to prevent the onset of collapse"

Rogers added that

"In young children, as well as in old persons with a tendency to rapid recurrence of collapse after an intravenous saline, slow continuous subcutaneous hypertonic saline (*without any sodium bicarbonate on account of its irritating properties*) may often be administered with advantage by means of a quart thermos flask to keep the fluid at a little above normal blood heat, such as 104°F [40°C] to allow for a cooling during passage through the tubing"

The contention of Rogers that under ordinary conditions the intravenous administration of infusion fluids is the method of choice for the treatment of severely affected cholera patients has been generally accepted.

Though on the whole the well known technique generally used for intravenous infusions is also adopted to treat cholera patients, a few points deserve special consideration. Workers not used to treating these dehydrated and collapsed sufferers, whose veins are often so little filled as not to be clearly visible may find it most difficult to introduce the needle of an ordinary infusion apparatus directly through the skin into the blood vessel chosen. This is particularly true of children, in the case of whom it often becomes necessary to select, in place of the veins usually chosen at the bend of the elbow some other vein such as the vena saphena on the thigh the vein in front of the malleolus internus on the ankle, or a vein on the dorsum of the hand.

Even in adult patients some workers found the difficulties of introducing the infusion needles directly through the skin so considerable that they preferred the "open method" of introducing an intravenous cannula after the vein had been laid bare by an incision in the skin. Recommending this procedure, Rogers (1921) described its technique as follows

"No anaesthetic is needed for this small operation The front of the elbow or other site chosen having been sterilized as well as circumstances permit, a piece of bandage is tied tightly with a slipknot around the limb above the position so as to distend the veins as far as possible. An incision 1 inch or more is made over the course of the vein which must be carefully exposed and dissected out from the surrounding structures before being opened A double strand of silk is now passed under the vein, and the

lower or distal part tied with one strand while the other is looped loosely round the upper portion ready to be tightened the moment the cannula is inserted. The vessel is then opened in the following manner. The superficial wall is seized with a fine forceps, and an oblique cut sloping upwards and backwards beneath the forceps is made with a pair of scissors through half the circumference of the vessel, thus forming a small flap which is held open by the forceps. The cannula is then passed beneath this flap with the other hand. After being passed in for about an inch, the remaining ligature is tightened around it. The vessel holding the solution having been previously filled and all air carefully excluded from the tubing by squeezing the rubber tube from below upward, and by allowing a full stream to run through before inserting the cannula, it only remains to remove the bandage round the limb to allow the fluid to pass into the vein."

A useful modification of this method devised by Chatterjee and Paul in which a curved surgical needle was used as director for the incision of the vein and the introduction of the cannula has been described and illustrated in a 1944 editorial of the *Indian Medical Gazette*.

The insistence of Rogers and some other authors on the use of the open method is rather surprising because in the experience of many other workers, e.g., a large majority of those in China, the difficulties of introducing the infusion needles directly through the skin into the veins were by no means insurmountable. This much simpler operation may be greatly facilitated by the use of adapters to connect infusion needles of a suitably small calibre with the rubber tubing of the infusion apparatus. Such adapters, as well as the cannulas used for the open method, should preferably be provided with stopcocks.

The question at what temperature the infusion fluids should be used for the treatment of cholera has been differently answered by the various workers. Describing the technique of this therapeutic method Nichols & Andrews (1909) stated that they first heated the flasks containing the infusion fluids to a temperature of 43 C (109.4°F), so that, as Rogers (1921) commented, allowing for temperature loss in the tube of the infusion apparatus the saline solution entering the vein would have a temperature of from 4°F to 6°F (2-3 C) above the normal body heat. In the opinion of Rogers, however, it was essential carefully to adjust the temperature of the infusion fluids to the rectal temperature of the cholera patients. If, as was the case in about 60% of the patients, the rectal temperature was between 97°F and 100 F (36°C and 37.8 C), infusion fluids having the normal body temperature ought to be used, while with a rectal temperature below 97°F (36°C) it was indicated to commence infusion with saline solutions warmed to 102°-104°F (39°-40 C). However, in Rogers's opinion the most important point was to use comparatively cool infusion fluids when the rectal temperature of the patients was high. Thus he stated:

"If the rectal temperature is over 100°F [37.8°C], the solution should be run at several degrees below normal, while if the temperature in the rectum exceeds 102°F [38.9°C] I omit to warm the solution at all, but give it at the temperature of the room, which averages over 80°F [27°C] in Calcutta."

Banerjee (1938) ascribing the feverish reactions observed in cholera patients after saline infusion mainly to an inappropriately chosen temperature of the fluids used, recommended that

"the temperature of the saline solution should be as many degrees above 36.7°C, as the rectal temperature is lower than 36.7° and vice-versa"

so that, for instance, in the case of a patient with a rectal temperature of 38°C an infusion fluid having a temperature of 35 4°C ought to be used

It would appear that modern writers were not quite so punctilious when making recommendations regarding the temperature of the infusion fluids for cholera treatment. Thus Napier (1946) considered it "a very unnecessary complication of administration" to warm, in the hot climates in which cholera epidemics usually took place the infusion fluids to body temperature. However this was advisable when the weather was cool On the other hand great caution had to be exerted when administering intravenous infusions to cholera patients with a rectal temperature of 101°F (38 3°C) or more so as to avoid hyperpyrexia Certainly the infusion fluids used under these circumstances should be at no more than room temperature

Henderson & Seneca (1951) while recommending that the temperature of the infusion fluid when actually entering the vein should never be less than 99°F (37.2°C) and ought to be two or three degrees Fahrenheit higher in any except febrile patients maintained similarly to Napier s statement that

"The possibility of adjusting each patient's infusion to his body temperature at the time, however attractive in theory is remote under the usual conditions of epidemic cholera."

The problem of determining the amounts of the infusion fluids necessary to restore the biochemical equilibrium of the cholera patients has engaged the attention of many observers

Nichols & Andrews (1909) relied in this respect upon clinical criteria, stating that, though on the average they injected about 1500 ml of saline solution at a time not more than 500 ml were used if this amount sufficed to give rise to a good and strong pulse On the other hand, amounts varying from 2 to 3 litres were sometimes necessary to produce this result The two workers admitted that

"It is not always easy to determine when the injection should be discontinued. In collapse, the fluid part of the blood is withdrawn and its organic contents concentrated. Now if we could recognize when we had injected just sufficient fluid to restore the normal ratio between the solid and fluid parts of the blood this might be the signal for stopping the injection going beyond this point might increase the fluidity beyond normal and set the current the other way"

Rogers (1921) considered observations on the pulse and the blood pressure of cholera patients to be an important means for determining the need for infusions not only when the sufferers were first seen but also when after initial treatment relapses were apt to occur At the same time, how

ever he stressed the paramount importance of determinations of the specific gravity of the blood for an assessment of the amounts of fluids which had to be administered He stated in this connexion that if in adult male cholera patients the specific gravity of the blood was 1 063 3 pints of saline had to be given while with a specific gravity of 1 064 4 pints were required and if the specific gravity of the blood had risen to 1 065 usually 5 pints of saline were needed [1] Rogers added that if necessary he had even given 6-pint infusions to advantage He recommended that in the case of markedly collapsed cholera patients it was as a rule advisable to give the infusions

"at the rate of 1 pint in five minutes, or 4 ounces per minute, but a careful watch must be kept for any sign of distress, especially in old people If severe headache or oppression in the chest with quickened breathing is produced, the rate should at once be much slowed. If a stopcock cannula is not available, this may be done by lowering the height of the vessel containing the fluid to only just above the body of the patient, or by partially clamping the rubber tubing."

If Rogers continued, administration of 3-4 pints of saline at the rate of 4 ounces per minute was insufficient to restore the pulse and dilute the blood to normal a further pint or two of saline might be given at the rate of ½ to 1 ounce per minute Further

"If the copious evacuations continue and collapse rapidly recurs after the pulse has once more been restored by a second rapid injection the flow may be continued at a very slow rate for several hours, if it appears to be doing good."

Rogers pointed out, however that, if hypertonic saline was used, continued infusions were rarely required and moreover were not successful in the case of severely affected patients.

The simple method used by Rogers for the estimation of the blood specific gravity was as follows

A series of solutions of glycerine and water were prepared the specific gravity of which determined with the aid of a urinometer represented every other degree from 1 040 to 1 070 From these stock solutions kept in stoppered bottles, amounts of a few drams each were filled into duly labelled, small stoppered bottles Estimations were carried out at the bedside

"by placing a small drop of blood, by means of a capillary tube, in the middle of one of the bottles of glycerine solution of about the specific gravity which is expected to be found. If it immediately rises, it is lighter than the fluid, and another drop is placed in a bottle of lower specific gravity or vice-versa, until the one in which it just floats for a second or two before sinking is found, or it has been noted to rise slowly in one and sink in the next one in which case the correct figure will be between those on the two latter bottles."

The opinions held by subsequent observers regarding the methods to be used to assess the need for and the amounts of infusion fluids to be given to cholera patients were rather divergent Some workers laid the main or

[1] According to Rogers, amounts of saline solutions not exceeding 3½ and 2 pints respectively sufficed for the treatment of (Indian) females and of 10-15-year-old children.

even the sole stress upon a clinical appreciation of the fluid loss. Thus Sellards (1944) stated

"The inspection of the patient, the character of the pulse, and the amount of fluid lost by the bowel serve as adequate guides for the frequency of the injections. Various tests have been devised for this purpose, such as the determination of the specific gravity of the blood. In the opinion of the writer fluid should be supplied freely before the specific gravity of the blood is increased beyond normal limits."

Other observers pointed out that in place of specific gravity determinations advantage could be taken of other methods of blood examination in order to assess the haemoconcentration developing in cholera However Awny (1948) who as quoted in the preceding chapter recently advocated this view stated that a close parallel usually existed between the values thus established and the clinical condition of the patients Ghanem & Mikhail (1949) found the same to be true when correlating the results of determinations of the plasma specific gravity with observations on the clinically manifest degree of dehydration The two workers furnished the following tabulation

Degree of dehydration	*Plasma specific gravity*	*Amounts of infusion fluids needed per 24 hours (litres)*
Slight (+)	1.025-1 030	6-8
Moderate (++)	1 031 1 040	8-10
Marked (+++)	1 041 1 050	12-40

In contrast to the workers just quoted other observers such as El Ramli (1948) and Chaudhuri (1950) maintained that determinations of the specific gravity of the whole blood were of value. The former author stated that it was his rule " to give 400 cc. of normal saline for every degree above 1060 that is, if the specific gravity of the whole blood was 1064 1600 cc. of saline were given " However some cholera patients were met with who showed signs of dehydration even though their blood specific gravity was 1 060 or less. As a rule intravenous administration of 1.5-3 litres of saline sufficed to rehydrate these sufferers The total amount of saline given by El Ramli to cholera patients in general apparently rarely exceeded 5-6 litres.

As pointed out by this author the following method was useful if no facilities for the determination of the blood specific gravity were available

" Empty the urinary bladder on first seeing the patient, by catheter and leave the catheter in or put it in every half hour Then stop the i.v saline or diminish its speed (according to the general condition of the patient) when the kidney starts to excrete urine."

It was necessary carefully to watch such patients so that intravenous infusion could be repeated if signs of dehydration reappeared However sufficient oral fluid administration usually obviated the need for further infusions.

Determination of the blood pressure was also a useful guide for the proper conduct of the infusion treatment A blood pressure which was not measurable or very low indicated the need for rapid rehydration (intra venous infusion at an initial rate of 2 3 litres per hour) but with improve ment of the blood pressure the rate of infusion was slowed down and saline administration was stopped if the blood pressure seemed to have reached a permanently satisfactory level However a close watch for relapses was essential

The patients in whom a high specific blood gravity was found even though their blood pressure showed no or only a slight deviation from the normal, needed only small amounts of saline

Henderson & Seneca (1951) recommended that, if no repeated determina tions of the blood specific gravity or of the haematocrit values could be made, an empirical adult dose of 2000 ml be administered for initial treat ment the same amount to be given again every 4-6 hours as needed. In the opinion of these workers the use of saline solutions for the treatment of cholera could be as liberal as desired Caution was necessary only when treating patients with a pre-existing cardiac affection or in the case of sufferers " who pass from dehydration to congestion as a result of treatment "

The US Army recommendation for cholera treatment quoted by Shattuck (1951) though admitting that the patients usually required relatively huge amounts of saline and that such treatment might have to be continued over a " surprisingly long " period advised caution because too much fluid was dangerous particularly if given after the blood had re gained its normal specific gravity A still more peremptory warning was issued by Napier (1951) who stated that

" the recent tendency has been to overdo the saline infusions. No more than the immediate requirements should be given it is probably better to judge by the patient s general condition, pulse and blood pressure than by any rule of thumb *vis-à-vis* blood specific gravity "

However if the blood specific gravity was taken as guide, the amounts of saline to be administered were in Napier s opinion (see also Napier 1946) as follows

Specific gravity	*Dosage of infusion (pints)*
1.058-1 060	1½
1.060-1 062	2 2½
1 062 1 064	up to 3 pints

Note The first pint could be given at the rate of 4 ounces per minute after this the speed of infusion was to be reduced to a pint in about 20 minutes or less, if the patient showed signs of distress.

The disquieting fact that injudicious administration of infusions to cholera patients may lead to the appearance of pulmonary oedema has been recently reaffirmed by Chakravarti & Chaudhuri (1954) who stated in this connexion that

" Genesis of pulmonary oedema in cholera is usually the outcome of a large quantity of saline given in a short time in an effort to restore the blood pressure of the recurrent collapsing cases. These cases would in all probability have terminated in irreversible shock as occurs in haemorrhagic shock (due to extreme depletion of the circulatory blood volume) Any amount of saline or other plasma expander fails to prevent this catastrophe Every restraint should be exercised in treating these cases with infusion because even if they survived the collapse, the excessive saline would probably kill them from pulmonary oedema and renal failure."

Chakravarti & Chaudhuri added

" The clinical guide for further infusion in these cases would be repeated estimations of specific gravity of blood and plasma. But estimation of plasma sodium, potassium and chloride, carbon dioxide combining power urea, etc., should also be done whenever possible to assess the exact state of electrolytic balance in the body before loading the system with more salt and water "

The two workers also pointed out that in cholera patients whose initial rehydration had been much delayed too prolonged a hypoxia could adversely affect the myocardium. Under these circumstances a lung oedema of the left ventricular failure type could develop even if no excess of saline had been administered No doubt such patients can also succumb to sudden heart failure if saline infusions are given to them too rapidly

Evaluation of the various infusion fluids

The comparative value of the various infusion fluids used for the treat ment of cholera may be outlined as follows

Alkaline solutions

In the course of his pioneer study on the alkali tolerance in cholera, Sellards (1910) found that treatment of two comparable series of patients with sodium chloride infusions and with alkaline solutions (sodium bicar bonate or sodium acetate) respectively gave the following results

Method of infusion	*Treated*	*Recovered*	*Died*	*Deaths from uraemia*
Sodium chloride	56	16	40	8
Sodium bicarbonate or acetate	55	27	28	1

It thus appeared that early treatment with alkaline solutions not only lowered the mortality from cholera, but " practically eliminated death from uraemia "

These favourable experiences have been confirmed by many subsequent workers, first by Rosenthal (1914) and later by Rogers who in 1921 commented thus upon the results he had obtained during the previous six years with this therapeutic method

" At first I gave a pint of this solution to every case coming in late with suppression of urine, and to all cases requiring a second further injection after the first. The resulting

reduction of the uraemic deaths by 70 per cent. during three years trial in 584 cases was so satisfactory that I now use 1 pint of the alkaline solution in every case requiring an intravenous injection and continue with the hypertonic solution to make up the full amount required. Thus, if 4 pints are indicated 1 pint of the alkaline solution is first given and 3 pints of the hypertonic one continued through the same flask and cannula as there is no objection to the two solutions being mixed. This is repeated with each injection as long as collapse or a high specific gravity of the blood indicates further hypertonic saline injections and the urine, if present, is still acid."

The value of this combined method of treatment for the abatement of acidosis and the prevention of uraemia is so obvious that as will be discussed below therapeutic schemes identical with or similar to that of Rogers continue to be followed by almost all modern cholera workers

Sodium chloride solutions

While the necessity of using saline solutions for the treatment of cholera is universally acknowledged opinions on at what strength these ought to be administered vary widely

Rogers (1921) in order to demonstrate the superior value of cholera treatment with hypertonic saline (later on combined with the administration of potassium permanganate and of alkalis) quoted the following Calcutta statistics

Period	*Method of treatment*	*Number of patients*	*Number of deaths*	*Percentage of recoveries*	*Percentage of deaths*
1895-1905	Saline intrarectally and subcutaneously	1243	783	37.0	63 0
1906	Normal saline intravenously	112	57	49 1	50 9
1907	Saline intrarectally and subcutaneously	158	94	40.5	59.5
1908-09	Hypertonic saline intravenously	294	96	67 4	32.6
1910-14	Hypertonic saline and potassium permanganate	858	222	74 1	25 9
1915-19	Hypertonic saline potassium permanganate and alkalis	1429	298	79.2	20.8

Further evidence supporting the value of hypertonic saline infusions for the treatment of cholera has been brought forward by many other workers. Particularly instructive are the records of some observers who were able to compare the efficacy of infusion treatment with either hypertonic or normal saline solutions during one and the same cholera epidemic Chun (1934) who had opportunities of making such comparisons at Harbin both in 1919 and in 1926 recorded in this respect the following mortality figures

Hospital	*Method of infusion*	*Mortality percentages 1919 outbreak*	*Mortality percentages 1926 outbreak*
Government Isolation Hospital	Hypertonic saline	14 11	17.2
Municipal and railway hospitals	Normal saline	54.5	34.5

"Genesis of pulmonary oedema in cholera is usually the outcome of a large quantity of saline given in a short time in an effort to restore the blood pressure of the recurrent collapsing cases. These cases would in all probability have terminated in irreversible shock as occurs in haemorrhagic shock (due to extreme depletion of the circulatory blood volume) Any amount of saline or other plasma expander fails to prevent this catastrophe Every restraint should be exercised in treating these cases with infusion because even if they survived the collapse, the excessive saline would probably kill them from pulmonary oedema and renal failure."

Chakravarti & Chaudhuri added

"The clinical guide for further infusion in these cases would be repeated estimations of specific gravity of blood and plasma. But estimation of plasma sodium, potassium and chloride, carbon dioxide combining power urea, etc., should also be done whenever possible to assess the exact state of electrolytic balance in the body before loading the system with more salt and water"

The two workers also pointed out that in cholera patients whose initial rehydration had been much delayed too prolonged a hypoxia could adversely affect the myocardium. Under these circumstances a lung oedema of the left ventricular failure type could develop even if no excess of saline had been administered No doubt such patients can also succumb to sudden heart failure if saline infusions are given to them too rapidly

Evaluation of the various infusion fluids

The comparative value of the various infusion fluids used for the treat ment of cholera may be outlined as follows.

Alkaline solutions

In the course of his pioneer study on the alkali tolerance in cholera Sellards (1910) found that treatment of two comparable series of patients with sodium chloride infusions and with alkaline solutions (sodium bicar bonate or sodium acetate) respectively gave the following results

Method of infusion	*Treated*	*Recovered*	*Died*	*Deaths from uraemia*
Sodium chloride	56	16	40	8
Sodium bicarbonate or acetate	55	27	28	1

It thus appeared that early treatment with alkaline solutions not only lowered the mortality from cholera, but "practically eliminated death from uraemia"

These favourable experiences have been confirmed by many subsequent workers, first by Rosenthal (1914) and later by Rogers who in 1921 commented thus upon the results he had obtained during the previous six years with this therapeutic method

"At first I gave a pint of this solution to every case coming in late with suppression of urine, and to all cases requiring a second further injection after the first. The resulting

Treating four cholera patients according to Massias s method Narayana Rao (1935) obtained excellent results He noted in particular that the sufferers began to pass urine a few minutes after they had received the concentrated saline injections

In a summary of further observations on this point by Banerjee (1942) in the 1941 report of the Indian Research Fund Association, it was stated that administration of 50-ml doses of a 20% sodium chloride solution to six collapsed cholera patients rapidly and effectively lowered the blood specific gravity and proved as useful as 3 pints of the usual hypertonic saline solutions Evidently the beneficial action of the concentrated saline solution was due to the drawing of interstitial fluid into the circulation However the injection of such concentrated solutions was apt to produce intense headache which, appearing during the administration persisted for some time

As far as the present writer is entitled to judge, the evidence brought forward above clearly proves that, in spite of assertions to the contrary made in recent years, administration of hypertonic saline solutions remains an eminently useful method of cholera therapy He is reassured to note that this opinion has been endorsed by as experienced an observer as Napier who writing in 1951 made the following excellent statement

> "During the last few years there has been a tendency to depart from Rogers hypertonic saline and alkali routine. Physiological considerations and the recent extensive studies in water and salt balance have led to this. It has been pointed out that hypertonic saline solution will only lead to a further call on tissue fluid which is obviously undesirable One can only refute these arguments on the ground of practical experience and anyone who has seen collapsed, pulseless and shrivelled patients recover their pulse, literally swell in front of one and fall into a comfortable sleep in a few minutes while hypertonic saline solution is being given will forget the theoretical considerations."

At the same time, however it would be erroneous to make a fetish of the hypertonic saline solutions instead of judiciously combining their use with that of sodium chloride solutions of lesser strength The modern concept of using a well balanced therapeutic scheme when attending cholera patients will receive due consideration in the concluding section of the present disquisition

Glucose infusions

While in the treatment of cholera fairly ample though not universal, use has been made of glucose infusions rather different views have been held in regard to the rationale of this therapeutic method Whyte (1913 1915) who was apparently the first to have added glucose to the saline solutions prepared for the treatment of severely affected cholera patients, did so in order to convey nutritive substances to them. Parenteral glucose administration was recommended for the same purpose by subsequent workers, Napier (1946) for instance, recommending that if this sugar

Similarly Robertson & Pollitzer (1939) recorded a mortality of only 7.33% among 150 cholera patients treated by them at Changteh (Hunan Province, China) with hypertonic saline infusions, followed if necessary by the administration of alkaline solutions, while out of 20 sufferers treated during the same epidemic in a missionary hospital with normal saline 11 (55%) succumbed. When the latter hospital went over to the initial administration of hypertonic saline, followed by infusions of glucose solutions made with normal saline, the mortality dropped to 14.3% (9 deaths among 63 patients). These observations, made in Manchuria and in the interior of China, stand in curious contrast to those in Shanghai where, it was claimed, treatment of cholera patients with normal saline gave fully satisfactory results.

The superior value of cholera treatment with hypertonic saline was also demonstrated during the 1947 Egyptian epidemic, Kamal and co-workers (1948) reporting a mortality of 24.1% in 245 patients infused with Rogers's solution as against a death rate of 33.7% in 579 sufferers given isotonic saline. It is curious to note that in both these series combination of the infusion therapy with sulfonamide treatment gave somewhat worse results than administration of saline solutions alone.

Inasmuch as in the opinion of some authors the administration of hypertonic saline solutions, instead of benefiting the cholera patients, is apt to prove harmful to them, it is important to note that some workers have made successful use of more concentrated sodium chloride solutions for the treatment of cholera. Thus Prašek (1914) reported that during an outbreak generally causing a mortality of about 50% there was a mortality of only 18%-22% among 40 patients to whom he had administered 0.5-l doses of a 5% sterile saline solution, warmed to 37°C, subcutaneously into the abdominal region. Additional proof of the efficacy of the method was the fact that a few sufferers, who by mistake were given a 1% instead of the 5% saline solution, did not improve. Early injection of 5% saline solution prevented the appearance of collapse in the case of three patients who had contracted the infection in the hospital.

De Raymond (1932) reported that he had been able to save 20 out of 22 patients suffering from choleriform diarrhoea (which usually caused a mortality of 60%-80%) through intravenous administrations of 10-20 ml of a 30% sodium chloride solution combined with intravenous Gonacrine injections once per day. He therefore recommended this method for the treatment of cholera. Massias (1933) actually found that intravenous injections of a 20% sodium chloride solution—if necessary, repeated after 12 hours and again on the following day—were of value for cholera treatment, lowering the mortality to 22%. Massias was not in favour of using 30% saline solutions, because these produced tachypnoea and arterial hypertension and thought that possibly 10% saline solutions might prove as efficacious as the 20% solution he had used.

In a personal conversation with Shattuck, which the latter quoted Amberson postulated that

"1 Plasma should be administered to all suspected cases of cholera, whether mild or severe, at the earliest possible moment without waiting for confirmation of the diagnosis.

"2. Administration of at least 500 ml of plasma is promptly followed by relief of symptoms of collapse, vomiting and diarrhea normal saline solution should then be administered intravenously to combat acute dehydration

"3 Thereafter fluids can be taken by mouth and sulfadiazine can be given by the oral route."

Napier (1946) mentioned that treatment of a few cholera patients with 2 pints of hypertonic saline, followed by 1 pint of plasma, had apparently led to complete success Nevertheless he maintained that "there are not the same indications for giving plasma in cholera as in shock"

Ghanem & Mikhail (1949) recommended the administration of plasma to cholera patients if their blood pressure was very low and if the specific gravity of their blood plasma was normal or below normal

A definite stand against the advisability of using plasma for the treatment of cholera was taken by Kamal and colleagues (1948) as well as by Chaudhuri and co-workers (1951 see also Chaudhuri, 1950) The latter observers stressed in this connexion that (*a*) no appreciable loss of plasma protein took place in cholera, and (*b*) it seemed unwise further to increase the viscosity of the blood of the sufferers through introduction of a viscous fluid.

In general agreement with these views, Mackie and co-authors (1954) maintained that in the treatment of cholera

"plasma and whole blood are seldom necessary and may be very harmful. They should be given only when specific indications exist."

There is no doubt in the mind of the present writer that this is the statement that deserves attention rather than the postulations of Amberson.

It has to be noted however that as quoted in the *Tropical Diseases Bulletin* (1957) Banerji (1955) claimed that with intravenous administration of plasma in doses of from 250 ml to 750 ml he had saved all but two of 45 collapsed cholera patients who had not responded to the usual infusion treatment.

Attempts made by this worker to use a cheap synthesized substitute (polyvinyl pyrrolidone) in the place of plasma proved successful only in the case of moderately severe cholera attacks but not for the treatment of sufferers who had been in a markedly collapsed condition for some time It would appear that Basu (1956) as well obtained rather disappointing results when administering this plasma substitute to 48 most severely affected cholera patients

Schemes of treatment

Though within recent years several schemes for the infusion treatment of cholera have been proposed, the present writer feels convinced that a

cannot be given orally or intrarectally up to a pint of a 5% glucose solution ought to be injected intravenously

Strauss (1915) on the other hand, advocated the use, for the infusion treatment of cholera, of isotonic (4 5%) glucose solution in place of sodium chloride solutions, because in his opinion the administration of the latter was contra indicated in the presence of kidney lesions. Gaertner (1915) expressed the hope that glucose infusions would lead to a dilatation of the renal vessels and an increased diuresis, thus lessening the danger of kidney complications. According to Takano and co-workers, Murayama (1916) actually found that subcutaneous or intravenous administration of 3% glucose solution to not too severely affected cholera patients was followed by an increased urine output. Recently Chakravarti & Chaudhuri (1954) recommended glucose treatment as one of the means of coping with a dangerously high potassium level in the plasma of severely affected cholera patients Finding that in spite of the usual presence of hyperglycaemia in the 22 such sufferers studied by them, glucose was well utilized Banerjee et al. (1957) also advocated intravenous administration of solutions of this sugar for the treatment of cholera.

Whatever the action of parenteral treatment with glucose may be, it appears that this forms a useful, though not indispensable adjunct to cholera therapy However in taking advantage of this method, over dosage must be avoided. The 1945 US Army regulations for cholera treat ment quoted by Shattuck (1951) stated in this respect that

"Dextrose to make 5 per cent solution should be added to physiologic or hypertonic saline, but no more than 50 gm of glucose should be given in 1 hour or 400 gm. in 24 hours. It is desirable but not essential, to add thiamin chloride 1 mgm. for every 25 gm. of glucose."

Plasma administration

An attempt to improve the results of cholera treatment by administering plasma to the sufferers in addition to saline infusions and "chemotherapy" (consisting of medication with sulfaguanidine sulfadiazine or penicillin, or of a combination of sulfadiazine and penicillin) was made by Amberson (1945) with the following results

Method of treatment	*Treated*	*Recovered*	*Died*	*Mortality (%)*
Plasma and chemotherapy	35	35	0	0.0
Chemotherapy only	277	274	3	1 1
Controls *	60	37	23	38.3

* With "no treatment, insufficient treatment, or supportive treatment only"

It is noteworthy that, in contrast to the other two groups, all the patients receiving plasma were in a serious condition with frank shock or collapse.

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a mortality of 11 % among the former and 23 % among the latter According to the *causes of deaths the two groups* could be classified as follows

	Cause of deaths				Total deaths
	collapse	*uraemia*	*pneumonia*	*other*	
Atropine-treated	3	4	1	3	11
Controls	9	5	6	3	23

The use of atropine for adjuvant treatment in cholera has been continued by many workers Napier (1946) recently again recommending it in an initial dose of $\frac{1}{75}$ grain to be repeated after about 12 hours should collapse persist stated the following in favour of this medication

" Atropine reduces all secretions, except renal secretion It therefore helps to conserve fluid and at the same time to reduce the tendency to oedema of the lung when saline is given. It also reduces irregular peristalsis and is a cardiac stimulant."

Adrenalin

Some use has been made of adrenalin in the treatment of cholera Naamé (1937) *claiming that he had recommended this therapeutic method* in 1911 on account of the similarity between the choleraic syndrome and *suprarenal insufficiency He advocated* the use of subcutaneous injections of the drug in 4-6-mg doses every 24 hours for some days, combined, as indicated, with infusion treatment.

Rogers (1921) stated that adrenalin

" may be given both in the rectal salines and hypodermically in 10 to 20-minim doses of the 1 in 1,000 solution every four to six hours. Intravenously in very high dilutions, such as 1 in 100,000 *it may also be of value in flushing the kidneys, but caution is necessary* in this use of the drug on account of the danger of the sudden high pressure thus induced on the already enfeebled heart, especially in elderly patients. The effect of adrenalin is also of *short duration, while it will constrict the renal as well as the other vessels.* For this reason pituitary extract is of greater service "

Recently Lahiri & Basu (1954) tried to use noradrenalin for the treatment of collapsed cholera patients but to judge from a review of their work in the *Tropical Diseases Bulletin* (1954) their results were by no means satisfactory even though in a part of the patients thus treated pulse and blood pressure improved immediately

Suprarenal cortical extract

This was used *by Lahiri (1945) intravenously* in 2 ml doses in 100 ml of 25 % glucose solution followed by saline infusion This combined treatment was found to be effective Chaudhuri (1950) also *maintained* that suprarenal cortical extract was useful during the collapse stage of cholera or if cardiovascular weakness persisted after rehydration.

discussion of their relative merits at the present juncture would prove confusing rather than instructive It seems advisable, therefore, to focus attention upon the procedure outlined by Napier (1946) which was based upon a scheme worked out by B C. Chatterjee and other physicians of the Campbell Hospital in Calcutta, an institution justly renowned in the history of cholera treatment.

As will be gathered from the tabulation on page 786, this scheme pre supposes the use of four different infusion fluids, namely hypertonic saline, normal alkaline saline alkaline hypotonic saline and 5% sodium bicar bonate solution. Whenever possible determinations of the blood specific gravity and the blood pressure should be made to ascertain the need for and the amounts of the first two solutions for initial treatment (see page 795 above) As stated by Napier (1951) hypotonic alkaline or sodium bicar bonate solutions ought to be administered in half pint amounts

The principle of the scheme recommended by Napier was that, since

" dehydration and loss of chlorides are the first pathological processes to be counteracted, hypertonic and alkaline saline should be given in the proportions 2 to 1 within the first 24 *hours of onset. Later acidosis develops, and during the next 24 hours, that is, from* 24 to 48 hours from the onset, the proportions should be reversed, and 1 part of hyper tonic saline with 2 parts of alkaline saline given. After 48 hours, acidosis will probably be the most prominent feature and hypotonic alkaline saline should be given. If however the specific gravity of the blood is not increased, but nevertheless the patient is suffering from acidosis, then the bicarbonate solution only is required, and about half a pint of this should be given."

Adjuvant Treatment

While most of the therapeutic methods recommended for adjuvant treatment in cholera deservedly attracted no more than ephemeral attention the following of them require discussion

Atropine

Though it is often stated that the use of atropine for the treatment of cholera was suggested by Lauder Brunton (1892 93) actually the history of this therapeutic method is much older as early as 1832 Viardin reported on attempts to cure the disease with belladonna and Hodgen (1866) an American worker advocated the cure of cholera with the aid of atropine and saline infusions.

Drawing attention to this therapeutic method, Rogers (1915) recom mended the subcutaneous administration of atropine to cholera patients in doses of $\frac{1}{100}$ grain for adults morning and evening during the acute stage of the disease The mortality in 75 patients given the drug as well as hypertonic saline infusions was 10 7%, whereas 18 (24%) of the 75 saline-treated controls succumbed In a further report on 100 atropine treated cholera patients and a control group of equal size, Rogers (1916a) recorded

a mortality of 11% among the former and 23% among the latter According to the causes of deaths the two groups could be classified as follows

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Pituitary extracts

The use of extracts prepared from the posterior lobe of the pituitary gland for the treatment of cholera seems to have been recommended first by Mueller (1916), who advocated their intramuscular administration in the case of collapsed patients and subcutaneous injections in the case of convalescents as a means of raising the blood pressure. As noted above Rogers (1921) preferred the use of these extracts to that of adrenalin on account of their longer lasting action as he added in 1952, the extracts ought to be used for the treatment of cholera in adult doses of 1 ml administered two or three times daily

Pituitary extracts were also recommended for cholera treatment by several other workers—e.g., by Napier (1946 1951), who stated, however that in their place desoxycorticosterone acetate (a synthetic suprarenal cortical extract) might be used in 20-ml doses to restore the pulse during the collapse stage.

As may be conveniently added Laigret (1955) found corticotrophin, the hormone secreted by the anterior lobe of the pituitary gland, in combination with saline infusions suitable for the treatment of cholera. Of 11 severely affected patients receiving this combined therapy four succumbed, one of them one month later after having shown initial improvement, the other three within a few hours. A total dosage of 75-100 mg of the hormone, administered gradually in the course of 24 hours was apt to influence the clinical picture in a spectacular manner

Cardiac stimulants

Though every effort has to be made to combat the circulatory failure as well as the fluid and electrolyte losses through a judiciously conducted infusion therapy the additional use of cardiac stimulants is not infrequently necessary Among the various drugs successively chosen for this purpose, caffeine sodium benzoate has been particularly recommended both by past workers (see for instance, Prašek, 1914) and recently again in the US Army regulations for cholera treatment (Shattuck, 1951)—presumably on account of its diuretic action Similarly Rogers (1952) was in favour of strophanthin because this drug, while raising the general blood pressure, was reputed to dilate the renal vessels

Treatment of uraemia

It is the ideal aim of cholera therapy to cut short oliguria or anuria and thus to prevent the development of uraemia through early and adequate administration of combined saline and alkaline infusion—the more so because in the experience of most workers the possibilities of coping with a developed uraemic condition are limited. The methods used for the latter

purpose in the past, such as hot baths, hot colonic washings application of hot fomentations or dry cupping of the kidney regions are naturally not fully effective Kidney decapsulation and incision or withdrawal of blood from the kidneys with the aid of a syringe have been tried by Rogers (1921) in a few desperately affected uraemic cholera patients but these heroic measures failed to save the sufferers.

While advising the administration of sodium bicarbonate to uraemic cholera patients by mouth intrarectally or if necessary in half pint doses of a 5% solution intravenously Chaudhuri (1950) stressed with great reason that

"In acute renal insufficiency it is important not to force too much fluid, as the resultant oedema may embarrass rather than encourage the renal function. It should be just enough to cover the losses It is also important that fluids by vein should be minimum. When the kidneys are out of action, oral intake ensures better adjustment of water-salt balance. It also keeps the patient from heart strain to which he is liable at this stage if fluids are given by vein No sodium chloride should be given in complete anuria except to replace what is lost with stool and vomit when a weak hypotonic solution should be enough."

It is remarkable that according to Henderson and co-workers (1948) the combination of an administration of 25-50 mg of testosterone propionate with the usual cholera therapy was an effective means of controlling post choleraic uraemia. While no less than 70% of the uraemic cholera patients treated in the same hospital in the usual manner succumbed there were five deaths only among 27 such sufferers to whom testosterone had been given as well one of them dying soon after the treatment had been commenced Up to the present however it does not seem that the above method has been tried by other workers

While Chatterjee in 1952 recommended treatment of post-choleraic uraemia by intravenous injection of ascorbic acid combined if necessary with intramuscular injections of an anti histamine compound into the kidney region Chakravati et al (1953) denied the value of this mode of therapy for the prevention and treatment of the uraemic state in cholera It is noteworthy however that recently Chatterjee (1957) again maintained the value of the method advocated by him in 1952 He stated in this connexion that

"In all cases where urination did not occur within two hours after infusion of saline solution, promethazine [an anti histamine preparation] was given intramuscularly in the kidney region. Patients who did not urinate within the following eight hours were given vitamin C 500 mg. intravenously In a few cases administration of both the anti-histamine drug and the vitamin C had to be repeated every twelve hours."

S C Lahiri et al. (1956) claimed to have obtained success when using daily gastric lavage with 12 pints of 10% cane sugar solution for the treatment of post-choleraic uraemia However one must agree with the reviewer of the article in question in the *Tropical Diseases Bulletin* (1957) that thus far the value of this mode of therapy has not been proved

Control of excessive vomiting

Various methods have been recommended for dealing with the excessive vomiting often observed during attacks of cholera gravis Thus Leo (1926) claimed to have had success with the oral administration of 2% sodium chloride solutions in 1-ounce doses every quarter hour to a total of five or six doses Some modern workers have advocated the use of cocaine, Henderson & Seneca (1951) for instance stating that

"some control of the vomiting can be secured with small doses of cocaine, such as 8 mg ($\frac{1}{8}$ grain) and it may be advantageous to administer this dropwise under the tongue in smaller amounts."

Chatterjee (1953b) stated that he had obtained excellent results with the administration of a proprietary drug containing promethazine and 8-chlorotheophylline in chemical combination. As noted before he advocated using this drug in combination with a remedy to check the diarrhoea and claimed that in this manner the need for infusion treatment could be obviated in patients with mild or moderately severe cholera attacks

Treatment of muscular cramps

Though the muscular cramps becoming manifest in cholera gravis are usually relieved through adequate infusion treatment, this may require some time so that special methods of treatment may have to be applied *ad interim* to lessen the often excruciating pains caused by the muscle contractions. In the opinion of some authors it is permissible to use small do es of morphine for this purpose, but most workers maintain with full justification that this or similar drugs should not be used in the treatment of cholera under any circumstances. It has been recommended instead that occasional whiffs of chloroform be given to the sufferers which while alleviating the pains in the contracted muscles cannot cause harm if over dosage is avoided

General Management and Diet of Cholera Patients

Though adequate treatment often results in rapid recovery cholera gravis must be considered an insidiously dangerous disease because (*a*) relapses may occur (*b*) uraemia may develop and (*c*) deaths due to acute heart failure may occur not only during the acute stage of the disease but even during convalescence It is necessary therefore to keep the sufferers in bed for several days, and, as long as they are in a collapsed condition, to use hot water bottles and other suitable means to keep them warm. The condition of their circulation, their surface and rectal tempera tures as well as their urinary output must be carefully watched so as to anticipate relapses, hyperpyrexia and uraemia which once they have

developed are difficult to treat Though permitted gradually to leave their beds cholera convalescents should not be allowed to resume their normal life before at least two weeks from the onset of the disease have elapsed Unfortunately however it is most difficult if at all possible to ensure compliance with these rules during widespread outbreaks and this is indubitably an important reason why the mortality from cholera is much higher than it would be under optimal conditions

In agreement with the statements of other writers, Chaudhuri (1950) has furnished the following diet scheme for cholera patients and convalescents which *mutatis mutandis* will also serve as a useful guide outside India

Stage of the disease	*Diet articles*
Active (24-48 hours)	Plain water or half normal saline in sips. Alternate drinks green coco-nut water soda water or glucose-saline flavoured with lemon juice.
Intermediate	Barley arrowroot or rice water with salt and sugar Whey or buttermilk may be added
Convalescents	Buttermilk and overboiled rice to which salt and sugar are added. Proceed cautiously to soft rice mashed green banana mashed potatoes, fish etc.

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Control of excessive vomiting

Various methods have been recommended for dealing with the excessive vomiting often observed during attacks of cholera gravis Thus Lieo (1926) claimed to have had success with the oral administration of 2% sodium chloride solutions in 1-ounce doses every quarter hour to a total of five or six doses Some modern workers have advocated the use of cocaine, Henderson & Seneca (1951) for instance stating that

"some control of the vomiting can be secured with small doses of cocaine, such as 8 mg ($\frac{1}{8}$ grain) and it may be advantageous to administer this dropwise under the tongue, in smaller amounts."

Chatterjee (1953b) stated that he had obtained excellent results with the administration of a proprietary drug containing promethazine and 8-chlorotheophylline in chemical combination. As noted before he advocated using this drug in combination with a remedy to check the diarrhoea and claimed that in this manner the need for infusion treatment could be obviated in patients with mild or moderately severe cholera attacks.

Treatment of muscular cramps

Though the muscular cramps becoming manifest in cholera gravis are usually relieved through adequate infusion treatment, this may require some time so that special methods of treatment may have to be applied *ad interim* to lessen the often excruciating pains caused by the muscle contractions. In the opinion of some authors it is permissible to use small doses of morphine for this purpose, but most workers maintain with full justification that this or similar drugs should not be used in the treatment of cholera under any circumstances. It has been recommended instead that occasional whiffs of chloroform be given to the sufferers which while alleviating the pains in the contracted muscles, cannot cause harm if over dosage is avoided.

General Management and Diet of Cholera Patients

Though adequate treatment often results in rapid recovery cholera gravis must be considered an insidiously dangerous disease because (*a*) relapses may occur (*b*) uraemia may develop and (*c*) deaths due to acute heart failure may occur not only during the acute stage of the disease but even during convalescence It is necessary therefore to keep the sufferers in bed for several days, and, as long as they are in a collapsed condition to use hot water bottles and other suitable means to keep them warm. The condition of their circulation their surface and rectal temperatures as well as their urinary output must be carefully watched so as to anticipate relapses hyperpyrexia and uraemia which, once they have

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Chapter 10

EPIDEMIOLOGY

Endemic Areas and Endemicity

Statistical evidence of the endemicity of cholera in the Bengal Presidency (now Bengal State) and adjacent areas, long suspected of being the principal endemic centre, if not the home of the disease, seems to have been furnished first by Bryden, statistical officer with the Sanitary Commissioner of India, in a series of four publications which began to appear in 1869 and were issued in collected form in 1874 As quoted by Yacob (1944) in Bryden's opinion the endemic area comprised " the western part of Assam, all the regions of lower Bengal and Orissa up to the low Rajnahal and Cuttack hills to the west of this basin as well as eastern Bihar " At the same time Bryden denied that cholera was endemic in any other part of India. However according to Swaroop (1951a) he " failed to give detailed data with regard to the Bombay and Madras Presidencies, hence his statements do not present a complete picture " The same may be said to hold true of the statement made by Koch at the 1885 cholera conference in Berlin that Bengal alone was the home of cholera.

In contrast to these observers, Bellew (1884) judging from a study of the records of the cholera incidence in India from 1862 to 1881 maintained that a state of endemicity existed not only in Bengal and the adjacent areas, but also in the interfluvial tracts of the Godavari Kistna and Cauvery rivers in Madras in the southern coastal districts of the then Bombay Presidency in Oudh and the southern Gangetic districts of the north-western provinces, and possibly even in part of the Punjab As pointed out by Bellew generally speaking the endemic areas appeared to be

" characterized by a low-lying alluvial soil, which is more or less supersaturated with ground water in a state of stagnation or but comparatively very slight motion, and which is subject to periodical inundations or water-logging by the seasonal floodings of the great rivers by which those areas are traversed in deltaic formation. These physical characteristics of the endemic areas are coupled with equally striking features characteristic of their climatic conditions, viz. with those of a moist and hot tropical climate, and they are among the most densely populated parts of the country "

The almost invariable validity of these general statements by Bellew has been conceded by all subsequent observers

Before continuing with a consideration of further studies on cholera endemicity in India attention has to be paid to contentions made by a considerable number of writers that endemic areas existed in other countries besides India. Rogers (1921) summarized that

"Apart from India, cholera is endemic in parts of the East Indies, Java having suffered as far back as 1629 It also occurs yearly in Southern China and the Philippine Islands. To the west the disease is so frequently carried to Persia and Arabia that it is difficult to say if it has become endemic in those countries or not. From 1851 to 1861 it was certainly present every year in Persia, but appears to have been frequently absent in subsequent years, so that it is probably not permanently located in that country The same remark applies to parts of South-East Russia."

Rogers opinions were not shared by Bernard (1936) who denied that cholera was endemic in Indochina, Indonesia or the Philippine Islands and doubted that the infection was permanently entrenched in China.

That such divergent opinions were expressed by different authors regarding the status of the various cholera affected areas is easy to understand, because there can be no doubt that in place of a permanent entrenchment of the infection in a given locality a state of temporary endemicity may exist. Attention to the latter was drawn by Gill & Lal (1931) for instance who pointing out that cholera sometimes persisted throughout the winter in the Himalayas and the northern part of the Punjab to become epidemic in the following spring, stated that

"There would thus appear a temporary form of endemicity which although lasting for one winter only is capable of causing a widespread epidemic mainly in the northern half of the Punjab in the following summer"

The existence of "secondary foci" (*foyers secondaires*) of cholera, where the infection persisted for three or four years, ultimately to disappear was postulated by Bernard (1936) In his opinion certain cholera foci considered as permanent, like those in Indochina and China, were actually due to such a temporary entrenchment of the infection

The validity of Bernard's contention was proved through observations made at Changteh situated on an affluent of the Yangtze river in Hunan Province central China. Robertson & Pollitzer (1939) were able to confirm the diagnosis of cholera in several patients seen in January 1938 as well as to isolate *V cholerae* from some samples of the Yuan river water and learnt from the local doctors that similar outbreaks of varying extent had taken place practically every winter throughout a number of years It seemed likely that these manifestations stood in causal connexion with the frequent summer epidemics occurring in that area as well as in the adjacent Yangtze valley and that thus the problem of cholera endemicity in the latter postulated by many authors, had been solved However when a few years later the present writer again stayed at Changteh to combat a

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It is also interesting to note that according to an account published in the 1941 report of the Indian Research Fund Association a spurious form of endemicity existed in the Tanjore district of Madras State which was due to differences in the seasonal incidence of cholera in the various parts of this region with the result that outbreaks were apt to commence in some of its parts at the time when they terminated in others

In the first of a series of three most important articles devoted to a statistical study of the cholera incidence in south west Bengal Lal Raja & Swaroop (1941) pointed out that the various districts of this area "present considerable heterogeneity in regard to their cholera experience and that there are also evidences of heterogeneity within the districts themselves" For a closer study of the endemiology and epidemiology of the disease it was necessary therefore to divide up south west Bengal into what the authors called "homogeneous cholera districts"

As discussed by Lal et al (1941) in their second publication the following steps were adopted for this purpose

"1 Taking the thana [local district] as a unit the total variability of cholera incidence has been split up into three variables, viz., seasonal yearly and residual by the method of analysis of variance.

"2. Contiguous thanas showing similarity in respect of different types of variation, mean cholera incidence and type of seasonal curve have been combined and the districts so obtained tested for homogeneity by trivariate analysis of variance Those satisfying tests of homogeneity have been constituted into cholera districts."

It was found that the size of the 19 cholera districts thus created varied considerably from single thanas to combinations of up to 20 such local administrative units

As stated in the third article of the series of publications presently under review Swaroop et al (1941) found that in 9 of these 19 cholera districts a state of endemicity existed whereas 10 experienced only epidemic cholera manifestations. Classifying the endemic as well as the epidemic homogeneous districts according to the degree of their epidemicity Swaroop and his co-workers obtained the following results

Category of districts	*Degree of epidemicity*					
	very high	*high*	*moderate*	*low*	*very low*	*total*
Endemic	3	—	—	1	5	9
Non-endemic	2	1	1	1	5	10

The interesting fact may thus be noted that cholera was but little epidemic in a majority of the endemic districts It is an intriguing question to what extent this absence of major cholera outbreaks was the result not of extrinsic conditions but of a herd immunity acquired during preceding epidemics Reference has to be made in this connexion to the following statement made long ago by Hart and his collaborators (1910)

"Within certain areas in India cholera is endemic especially in the country of the Lower Ganges. If however we examine carefully the incidence of cholera within the

plague outbreak, he was unable to find any evidence of the continued existence of cholera and, as far as is known the region continued to remain free from the infection.

Again turning attention to the problem of cholera endemicity in India, reference has to be made first to large-scale investigations undertaken by Russell & Sundararajan (1926 1927) As the former summarized at the 1927 Conference of the Far Eastern Association of Tropical Medicine in Calcutta (Russell 1928) a study of the cholera mortality over a long period of years had made it possible to divide the provinces of India into three groups

"I. The first group includes the provinces of Assam, Bengal, Bihar and Orissa and the United Provinces, where more or less uniform figures are registered annually and where the average incidence is high. These areas are very likely to be endemic in nature.

"II. In the second group are included the Central Provinces Bombay Presidency and the Punjab and North West Frontier Province where sudden peaks in cholera incidence occur at irregular intervals. These areas are normally free from cholera epidemics and infection is probably always brought in from outside.

"III. The Northern and Central Districts Groups of Madras Presidency are epidemic areas whilst the Southern Districts Group which presents a more uniform incidence, might almost be included in Group I as an endemic area."

Russell added that the differentiation of the areas of India into epidemic and endemic groups had been confirmed by various other statistical methods His and Sundararajan's investigations had established that cholera tended to recur repeatedly in river deltaic tracts, the main endemic areas of India including the delta areas of the Ganges, Brahmaputra and Cauvery rivers The outbreaks commenced in the towns or villages lying on the banks of these and other rivers the infection then rapidly and systematically spreading down the waterways "Moreover" Russell added,

"there is no question that, in endemic areas, cholera spontaneously appears, year after year in the same villages and towns In other areas, *per contra*, it is necessary for other favourable conditions to be present before cholera becomes diffused, e.g. overcrowded and insanitary conditions associated with religious fairs and festivals."

Rogers (1926 1928) studying the incidence and spread of cholera in India, claimed that a state of endemicity existed not only in Lower Bengal, Orissa and Assam, but also (*a*) in the extra-deltaic western divisions of Bengal and the north-easternly sub-Himalayan divisions of the United Provinces (*b*) the extensive low lying districts of South East Madras (suspected by Russell & Sundararajan) and (*c*) a small low alluvial district of the North Konkan districts of Bombay Presidency (now Bombay State) lying in an area already incriminated by Bellew (1884) Rogers admitted however that the last three endemic areas differed from the "hyper endemic" area of lower Bengal Orissa and Assam "in that, although cholera is never absent for a whole year yet the rate per mille not very rarely falls to less than one tenth of the average rate"

of the observation period It is of interest that in this manner it became possible to establish the probable formation of a new endemic focus in the Bombay Presidency (now Bombay State) Otherwise however the results of these observations were in agreement with those referred to above

Comparing the various regions in which endemic areas were expected to be present, Swaroop noted that

"1 All endemic areas are located generally around rivers

"2 All these tracts lie in areas of high population density

"3 All of them lie in low-lying lands, i.e. none is more than 500 feet [150 m] above sea level

"4 All these tracts lie in areas of high absolute humidity"

There can be no doubt that these and other conditions usually prevailing in the endemic areas are rather favourable for the persistence of cholera and at the same time render the implementation of measures to control the infection most difficult. This is true in the first place of the water supplies because it is an almost or even quite impossible task to dig satisfactory wells in the low lying and water logged endemic areas, while owing to technical and financial reasons it is as a rule not within the realm of practical politics to provide pipe water throughout the often extended districts. Therefore the people densely populating them even if they possess a knowledge of the elementary rules of hygiene—which is an exception rather than the rule—must usually draw their drinking water supplies from the surface-water courses which because they also serve for all other household purposes and even as sewers are grossly contaminated and thus apt to convey any cholera infection present. Facilities for the rapid recognition and the isolation of cholera patients are likewise poor particularly during the frequent periods of high water level or of floods.

These and most other conditions prevailing in the endemic areas appear to be so favourable for the spread of cholera that the question to be asked is not why the infection persists but why it does not constantly cause wide spread epidemics As has been alluded to above it is probable that the development of a herd immunity in the endemic areas is apt to lead to a kind of equilibrium between the causative organisms and the host population Factors like food shortages which lower the resistance of the people to cholera, and more still, a seasonal influx of labourers or the arrival of other susceptible persons presumably play an important role in the recrudescence of epidemic manifestations

Epidemics and Epidemicity

Origin of epidemics

Cholera epidemics may arise either in endemic areas where as has been discussed above the infection, fostered by particularly favourable condi

endemic area, it becomes obvious that, although in every district deaths from it may be reported every year and every month in every year still the incidence of this mortality is by no means evenly distributed even within the endemic area cholera wanders about, one village after another being attacked and then left at peace for a time. It seems as if there were the same tendency for these outbursts to die down within the area as there is outside it "

For a further study of the problem of cholera endemicity in India Swaroop (1951a) computed for each cholera affected district the average mortality rate from the disease for the 10 years with the lowest mortality during the period from 1901 to 1945 and used the magnitude of this average as a rough measure of endemicity He emphasized, however that he applied this term in a purely relative sense postulating the existence of highly endemic or moderately endemic districts and of districts without or with a low or doubtful endemicity only as far as the mutual relations of the various areas were concerned. Still, Swaroop maintained that though his analysis did not demarcate the endemic areas in any absolute sense it served to exclude tracts of the country in which endemic zones of cholera could not be expected to lie

According to these observations the principal regions in which cholera showed a tendency to persist at high level were the following

" (1) In Bengal at the lower reaches of the Hooghly river i.e. in the delta formed by the Hooghly the Damodar and the Rupnarayan.

" (2) At or around the confluence of the Ganges and the Brahmaputra and the Meghna and in the lower reaches of their delta including that of the Madhumati

" (3) In Orissa in the deltaic zone of the four rivers, viz. the Mahanadi the Brahmani the Baitarani and the Subnarekha.

" (4) In northern Assam in the Brahmaputra valley

" (5) In Bihar in and around the Patna and Gaya districts, where the Sone, the Gandak and the Gogra join the Ganges.

" (6) In the U.P in the low-lying river basins beginning from the junction of the Ganges and the Jamuna at Allahabad and extending through Banares to the points where the Gumti and the Sone join them.

" (7) In South Madras in the Cauvery delta.

" (8) Possibly also in North Madras in the delta formed by the Kistna and the Godavari rivers and around the Colair lake." [1]

Swaroop also determined the average periods in months during which the various affected districts had been free from cholera during the past 30 years. The results obtained in this manner tallied well with those arrived at with the aid of the determinations mentioned above

To guard against the possibility that the above mentioned observations extending over periods of 45 and of 30 years respectively might fail to give a correct picture of the cholera endemicity recently prevailing in India, determinations were also made of the length of the weekly periods during which the various districts had remained free from cholera during the last 10 years

[1] A valuable special study on the endemiology of cholera in Madras has been published by Swaroop (1951b).

In the second type of outbreak, the single manifestations did not show a uniform distribution, on the contrary, the infection became entrenched in foci In these not many fell ill simultaneously, but infections occurred, as it were in chains, often standing in causal connexion with one another Secondary foci could form not only in other precincts but also in adjacent communities

Koch admitted that it was not invariably possible to demonstrate how the chains of infection, characteristic of the second type of cholera epidemic were formed For, besides severe slight and often unrecognized cholera attacks developed, and the patients were infectious not only during the manifest stage of the disease but also before and after this Moreover,

" infection was by no means always derived directly from the cholera patients but still far more frequently was produced indirectly through linen, clothes, beds, foodstuffs, insects, etc." [Trans.]

It was possible nevertheless to study the spread of the infection in sparsely populated rural areas, but this was most difficult in larger towns particularly because there cholera spread mainly among " the lowest closely herded together and constantly fluctuating strata of the population reaching but rarely the better situated "

The lucid statements of Koch quoted above, which indeed one might reprint with but little modification in a modern textbook on cholera, have been accepted by most subsequent observers

Owing to the great differences in the conditions prevailing in the various cholera affected areas, the comparative frequency with which outbreaks of either the first or the second type take place shows marked variations, explosive epidemics due to a contamination of major water supplies pre ponderating in some areas and protracted outbreaks in others It is important to note in this connexion that explosive cholera manifestations may take place in villages as in urban communities Thus Gill & Lal (1931) who were able to collect on this point large-scale statistics in the Punjab found that out of a total of 298 outbreaks in towns eight exhibited definite explosive characters, whereas there were 60 epidemics of this type among a total of 2917 village outbreaks

Climatic influences

Early observations

Dealing in his classical work on cholera with " the bearing of meteorological influences upon the spread of cholera " Macnamara (1876) took a determined stand against the postulation of many of the early observers that the prevailing winds exerted a direct influence in propagating the infection He quoted in this respect a statement made by the Bengal Medical Board on the 1817 18 epidemic, saying that the members of the Board while

tions, is constantly present, or in localities ordinarily free from the disease Most modern observers agree that such invasions of hitherto cholera free areas are the result of importations of the infection by means which will be discussed later in this chapter It is, however curious to note that the idea of an autochthonous origin of cholera outbreaks which had been amply discussed before the detection of *V cholerae* was afterwards supported by claims made by a few workers in regard to the possibility of a transmutation of cholera like vibrios into the true type the thus "regenerated" vibrios becoming capable of starting cholera manifestations in man However when the evidence brought forward in this respect was exhaustively discussed in Chapter 4 the conclusion was reached there that

"there is no convincing evidence to show that such transmutations take place under natural conditions and that consequently cholera-like vibrios or cholera vibrios which had lost their agglutinability with the usual specific sera form a reservoir from which epidemics may be produced *de novo*"

Types of epidemic

As first exhaustively described by Koch (1893) two main types of cholera epidemic may be distinguished—namely (1) an explosive type apt to end as abruptly as it started and (2) a protracted type of outbreaks which commence slowly and afterwards follow an insidiously prolonged course. Koch was however careful to point that often a combination of these two types of cholera outbreak became manifest. "Thus" he said

"particularly the first type, which appears mostly at first in pure form later becomes combined with the second type and finally altogether passes over into the latter It also occurs that a local epidemic begins with the second type until accidentally the infectious material finds its way into the water and then, according to the system of water supply produces small circumscribed explosions or suddenly produces infection in a whole precinct, occasionally even in the whole community" [Trans.]

Dealing in a detailed manner with the two types of cholera outbreak, Koch stated that in the first type of epidemic the instances of infection were distributed in a fairly even manner over the whole community affected without any immediate connexion between the individual manifestations. Such a uniform distribution of the infection, he continued could be produced only through a vehicle capable of acting simultaneously upon all or at least most of the inhabitants of the locality concerned, for instance, the air water soil or foodstuffs. Koch maintained, however that

"so far it has not been possible to demonstrate a role of the air soil or foodstuffs in the explosive cholera outbreaks. Likewise insects, which have been suspected with reason, cannot come into question, because cholera explosions occur not rarely during the cold season when a transmission through insects is out of the question Thus only the water remains and that this can be the actual vehicle of the cholera germ not only for single groups of the population of a community but for whole communities and even large cities, has been proved by past epidemics and quite specially by the present cholera outbreaks in Hamburg, Altona and Nietleben." [Trans.]

Later observations

Subsequent observations on the influence exerted on cholera manifestations by climatic factors may be discussed under the following headings

Temperature The contention of the early writers that as a rule cholera becomes epidemic during the warm season of the year and declines when cold weather sets in has been fully confirmed through further observations To quote an example Bernard (1936) recorded that in Indochina the disease was most rampant during the hot and dry season (April June) The situation again became aggravated after the cessation of the rains during September to November but a marked decline of cholera occurred during the period from December to February the coolest season in Indochina. The frequency of cholera cases thus stood in inverse proportion to the temperature as well as the precipitations (*chutes d'eau*)

Similarly to these and other observations Takano and colleagues (1926) summarized that August and September when importations of cholera into Japan were most likely to take place

"are the months in which epidemics reach their peaks. The epidemic begins to subside gradually in October and November and practically ceases in December"

Limited winter epidemics were reported in Japan upon two occasions only (1886 and 1917) but if the warm weather outbreaks started late, sporadic cholera manifestations were frequently observed during the first two months of the following year Thus as Takano and his associates admitted

"The existence of winter cholera in Japan indicates that the cholera vibrio is able to survive the winter But it is very rare that the cholera vibrio becomes the source of another epidemic after surviving the coldest weather of February No such instance has occurred since 1900"

Generally speaking however a recrudescence of epidemics after the sporadic subsistence of cholera throughout the winter not only occurs in the endemic areas but has also been observed by no means rarely in localities subject solely to epidemic inroads of the disease An interesting example of the latter kind was met with by the present writer during the period 1939-40 in the northern part of Szechwan Province in China, where the winters are quite severe The region in question had been quite heavily involved in 1939 which was a bad cholera year for China in general. As confirmed by a survey made in 1940 sporadic cholera cases continued to occur during the winter 1939-40 The spring of the latter year being unusually dry the infection soon flared up and a further serious outbreak resulted However though cholera thus temporarily persisted it did not become permanently entrenched in the area

The question why cholera usually shows a markedly greater tendency to spread during the warm seasons of the year than during cooler or cold

" they hesitated to express an opinion as to the nature of the apparent connexion between cholera and the easterly wind expressly stated their belief that of all the predisposing causes to cholera, the one most frequently and unmistakably in operation was alternations of heat and cold combined with rain, or a very humid state of the atmosphere "

Evidently being in accord with the latter contentions, Macnamara laid down the general rule

" that cholera will not extend during the cold of a European winter or even of our Punjab cold season "

It was true that cholera outbreaks during the winter had occasionally been observed in Europe e g., in the Polish army in 1830-31 But these exceptions did not invalidate the rule that a drop of the atmospheric temperature to 50°F (10°C) impeded the spread of cholera, especially in dark and gloomy weather whereas the progress of the epidemics was stopped when the temperature fell below 40°F (4 4°C)

At the same time Macnamara emphasized the important role of atmospheric moisture as " a necessary element for the development of the disease "

Regarding the role played in this connexion by the rain, he pointed out that in Bengal

" cholera is at its height every year in March and April and again in September and October and these are the very months in which we get heavy downpours of rain, washing the surface soil and its contents into the wells and tanks from which we procure our drinking water these storms are generally followed by intensely hot days. As soon as the regular rains set in, and we get a more or less continuous downpour for some three months, cholera ceases for the time and in fact until the close of the year when it breaks out again in the stormy weather which, with intervals of intensely hot days, succeeds the rain."

In the north western parts of India, the moisture-laden south west monsoon promoted the spread of cholera brought in from Bengal through boats sailing up the Ganges But as soon as the rains ceased and the dry west winds of the upper provinces set in, cholera began to decline and as a rule, remained in abeyance during the entirely dry period lasting from the end of September until the onset of the monsoon in the following June

The rarity of cholera outbreaks in winter is well illustrated by statistics of Hirsch (1883) according to which out of 920 epidemics occurring outside of India only 42 started or became prevalent during the cold season as against 261 in spring, 496 in summer and 121 in autumn

Sticker (1910) laid stress on the fact also noted by subsequent observers that cholera epidemics were particularly apt to occur in years during which an unusual drought prevailed Thus it had been shown by Wolter (1898) that the cholera periods in Hamburg from 1831 to 1873 invariably fell in dry years. The 1892 93 outbreaks in that city also occurred during a dry period between the wet years 1888-91 and 1894-98 the year 1892 being quite exceptionally dry

the Punjab for instance the latter was "nearly as high in the minimum cholera months of December to February as in the maximum ones of the rainy season in July" Therefore Rogers insisted there remained only

"the absolute humidity or aqueous vapour pressure which is measured as air pressure in terms of the length of a column of pure mercury at temperature 32 and is obtained from observations of the wet and dry bulb thermometers by means of special tables "[1]

Rogers admitted that no relationship existed between a high absolute humidity and cholera incidence but maintained that

"when we turn to the months of low absolute humidity we find that in every area in which this reading falls below 0 400 during the cold weather months, cholera at the same period falls to a very low rate as in Bihar the United Provinces, Central Provinces and North Deccan and altogether disappears in the Punjab"

"Still more significant" Rogers continued, "is the fact that the winter decline of cholera in Assam and Lower Bengal in January and February immediately follows the lowest absolute humidity in December to February of from 0 425 to 0 475 and the mortality rises once more in these areas with the much increased absolute humidity in March Equally close is the relationship to increasing cholera prevalence in North-western and Central India "

As further postulated by Rogers the autumn decline of cholera in the latter areas also coincided with a fall of the absolute humidity below 0 400 thus

"completing the evidence of the closest association with that degree of dryness and falling cholera mortality and indicating that this condition is unfavourable to the continued survival of the infective agent outside the human body in sufficient quantity to keep up the epidemic prevalence of cholera over large tracts of country"

The validity of Rogers s conclusions was upheld by a number of subsequent observers such as Chun (1933) in China, Khalil (1948b) in Egypt Yu Wei (1949) in Shanghai and Banerjea (1951) in the United Provinces (now Uttar Pradesh) of India However disagreement with the findings of Rogers was recorded by some other workers, like Dunn & Khan (1928) Gill & Lal (1931) and particularly by Russell. Referring to Rogers s initial work in a paper read at the 1927 Congress of the Far Eastern Association of Tropical Medicine Russell (1928) stated that

"The arguments brought forward in favour of an absolute humidity figure of 0 40 seem to be based on broad generalizations. With all due deference, it is suggested that conclusions of this kind cannot possibly be reached without submitting the available data to detailed statistical analyses and it does not seem that such methods were employed"

Russell stressed that relative instead of absolute humidity was of epidemiological importance but maintained at the same time that the clue to the cholera problem was not to be found in any individual climatic factor

[1] As defined by Chun (1933), absolute humidity is the weight of aqueous vapour in the air measured in terms of its mercury tension, so that low readings indicate both dryness and low temperature and vice versa In order to find absolute humidity one must first know temperature and relative humidity and then look p water vapour tension tables

periods is rather involved. At first glance it would be tempting to ascribe this difference to a more prolonged survival or even a multiplication of *V cholerae* in the surface water supplies or on substrates like fruits and vegetables at a high temperature Actually however as will be gathered from the relevant data quoted in Chapter 3 the length of survival of this organism under these circumstances was apt to show a decrease rather than an increase *pari passu* with an increase of the ambient temperature. Other factors must therefore be responsible for the increased frequency of cholera during the warm season. As recognized by Flügge in 1893 of great importance among these is that prevailing hot weather leads to an increased consumption of raw water and other cold drinks as well as of cold foods like fruits, salads and jellies apt to be contaminated with *V cholerae* There can be no doubt that the frequent prevalence of flies during the summer greatly facilitates such contamination Moreover as pointed out with great reason by Flügge, the consumption of the raw drinks and foods is bound to lead to the frequent appearance of gastro-intestinal affections caused by other species of micro-organisms, which in their turn lower the resistance to cholera infection.

A further postulation of Flügge was that the lowering of the level of rivers and other surface waters as well as of the ground water in the wells, apt to occur in late summer and autumn, might be of importance because if such waters became contaminated with cholera vibrios, their vibrio content would remain high and possibly also because the organisms could thrive better in concentrated waters containing a large amount of organic matter As maintained by Jolly (see Chapter 3 page 183) the survival of *V cholerae* in surface waters is possibly also governed by changes in the reaction of the water which might be favourable only at certain seasons of the year

Humidity Though many of the early observers had become convinced of the existence of a close relationship between a suitably high atmospheric humidity and the spread of cholera, it was only about thirty years ago that large scale studies on this subject were made by Rogers (1926, 1928) and by Russell & Sundararajan who embodied the final results of their exhaustive investigations in a research memoir published in 1928

Rogers, studying the relation between climate and cholera incidence in India, found that rainfall alone did not account for the seasonal distribution of the disease for the latter was at its minimum during the south-west monsoon in Assam and Lower Bengal, but at its maximum during the same season in other parts of the subcontinent, such as the Punjab

The mean temperature showed a closer relationship to the cholera incidence, since the disease was at its minimum during the winter season in the Punjab, the United and Central Provinces and the Deccan area of Bombay but the same was not true of Lower Bengal Determinations of the relative humidity also failed to furnish universally valid clues, since in

Long term periodicity

Evidence indicating that cholera manifestations in addition to showing a seasonal incidence, might also exhibit features of a long term periodicity was adduced by some of the earlier observers Thus as summarized by Russell & Sundararajan (1928)

"Bellew in 1884 produced statistics from every province in India, relating to the period 1862 1881 in order to prove that the disease appeared in triennial waves. He attempted to show that cholera tends to run a definite course of revival, decline and subsidence in the successive years of each triennial cycle"

In agreement with Bellew s postulation Koch stated at the 1885 cholera conference that, as shown by the mortality figures from 1870 to 1883 in Bombay Province the fatalities from cholera reached a peak every third year while the mortality was remarkably low during the following two years. The only exception to this rule occurred in the period immediately following the cholera year of 1875 when—owing no doubt to the influx of many half starved people during the famine then prevailing—the mortality from the disease remained high Koch felt convinced that this periodicity of cholera was due to the development of an immunity in the people who had survived attacks of the disease

Attention to a periodical recrudescence of cholera in India was again drawn by Russell (1925) who in a note published in the *Lancet* stated that he had applied the method of periodogram analysis to study the incidence of cholera deaths in Madras Presidency for a period of 25 years and had found evidence of a six year periodicity He afterwards established that the cholera mortality figures also reached peak values at intervals of 72 months in many other parts of India suffering from epidemic invasions of the disease

Rogers (1926) asserted, on the contrary that

"from a study of the tables I have worked out during the last twelve months of the cholera rates per mille over 200 districts and forty-five divisions of India, for a period of forty-five years, I am unable to trace anything like a three year up to a six year cycle if a long period of time is studied."

He felt certain therefore that Indian cholera outbreaks occurred at irregular intervals

However Russell & Sundararajan (1928) again claimed on account of their exhaustive statistical studies that cholera in India did show a long term periodicity Commenting upon these findings Russell (1928) made the following statements at the 1927 conference of the Far Eastern Association of Tropical Medicine

"Periodicities of longer duration while not obvious, have been demonstrated by the application of the periodogram method used by Brownlee [1919]. By this means, it has been found that, in nearly all the areas where cholera is epidemic, waves of the disease recur once every five to six years, whilst in the endemic areas a 4-5-years periodicity

Analogously Russell & Sundararajan (1928) analysing the results of their exhaustive statistical studies on the epidemiology of cholera in India, concluded that

"The association of high relative humidity with high temperature, accompanied by intermittent rains, forms the most favourable atmosphere for the development of the disease. The presence of endemic centres from which epidemics spring at short intervals is also a fact which must be accepted. No single factor however can be held responsible for the periodic waves of the disease and it must be recognized that these waves are preceded by conditions too complex to admit of complete solution with the aid of available data. Individual susceptibility foci of infection, favourable atmospheric conditions, fairs and festivals, carriers, insanitary habits, all play their part in a manner which defies analysis."

As far as the present writer is entitled to judge the great import of this statement cannot be overrated

Rainfall In agreement with observations by Macnamara (1876) quoted above Koch stated at the 1885 cholera conference that (*a*) even though the period from February to April, during which the incidence of cholera at Calcutta increased was generally a dry season, occasional heavy downpours of rain were by no means rare and (*b*) according to the local practitioners such rainfalls were invariably followed by a deterioration of the epidemic situation This was not surprising because the rains were apt to wash cholera infected faeces and other contaminated materials into the "tanks" (or ponds) serving as sources of water supply in the foci The danger of a heavy contamination of the tanks during the rainy seasons was considerably less because the infective materials then introduced were apt first to become diluted and finally to be carried away If on account of the absence of rains the level of the tanks became low a dangerously high concentration of cholera-contaminated materials introduced by bathers or through the washing of infected clothes or other objects might result.

The contention of Koch that, owing to variances in their duration rainfalls were apt to lead either to an exacerbation of cholera outbreaks or to a decline or even cessation of the epidemics has been confirmed through numerous subsequent observations It has to be noted however that prolonged rain does not invariably exert an inhibitory influence in some areas the epidemics continuing or even starting during rainy seasons

The early observations that cholera manifestations, if they arise after or during periods of exceptional drought often become particularly dangerous has likewise been reliably confirmed. At such times the people are forced to make use of the scanty water supplies remaining available, however unsuitable or even repulsive they may be If cholera becomes recrudescent or is imported, contaminations of these sources of water supply at which the people concentrate, is easily possible and once such infections have taken place, a rapid spread of the disease is well nigh inevitable

If consideration is given to the discrepant statements recorded above it is impossible to support the earlier claims that cholera epidemics regularly show a long term periodicity At the same time, however, Russell (1928) was certainly right when insisting that observations on this point made in individual cholera-affected areas may be eminently useful for directing the main efforts of the workers to potential danger spots

Forecasting of epidemics

As far as could be ascertained a successful attempt to utilize observations on past cholera manifestations for forecasts of future outbreaks was first made by King. Commenting upon Russell s 1925 article in a letter to the editor of the *Lancet* King stated that the statistical method described in the article

"was already employed by one of his predecessors in office in 1894 and formed the subject of a paper read before the first Indian Medical Congress This was illustrated by a diagram founded on the figures for a decade It inhibited [sic] monthly periodicity so markedly as in after years to justify it being termed the Madras Cholera Clock "[1]

Russell & Sundararajan (1928) paid a just tribute to King s pioneer work, stating

"that his cholera clock continued to keep good time and due acknowledgment must, therefore be made to that distinguished sanitarian for the clue on which most of the work to be described has been based."

As already alluded to, in the opinion of Russell (1925)

"the mere knowledge of this six-yearly periodicity of cholera has enabled the public health department in Madras Presidency to control its preventive work in connexion with the disease and has prevented waste of effort in unnecessary directions and at unnecessary times"

Nevertheless Russell & Sundararajan (1927) in order to forecast cholera epidemics resorted to the elaborate method for the early detection of epidemic trends devised by Bundesen & Hedrich (1925) These two workers had found that the expectancy or median incidence of an infectious disease during a period of from five to nine previous years was apt to serve as a reliable standard for gauging the present incidence of the disease and that the " epidemic index " or ratio of the current incidence to the expectancy was an approximate barometer of the fundamental epidemic trend the index often rising a number of months before the approach of an epidemic could be detected with the aid of the ordinarily used methods

Applying the new method in a somewhat modified form for a study of cholera incidence in India, Russell & Sundararajan established that by means

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[1] See Fig. 21

is most probable. In every case the periodograms show that cholera tends to run a more or less definite course of revival, decline and subsidence in each cycle of years. This phenomenon has been demonstrated further by the epidemic indices curves relating to the different areas of India [Russell & Sundararajan, 1927]."

Referring to Koch's postulation that an immunity developing in the survivors from cholera attacks accounted for the periodicity of the outbreaks, Russell stated

"Probably other factors have equal significance, but whatever influences may be at work, it is certain that fore-knowledge of the probable advent of a periodic peak in the incidence of the disease would go far to prevent waste of effort in unnecessary directions and at unnecessary seasons. In Madras, we have for three years past made use of that knowledge with very considerable success."

Further statements made in regard to the cyclical incidence of cholera epidemics may be summarized thus

Author	*Findings*
Chun (1935)	Stated at the 1934 conference of the Far Eastern Association of Tropical Medicine that "in order to ascertain if there was any periodicity in the cholera epidemics in Shanghai, a chart of the annual cholera deaths during the period of 49 years (from 1886-1934) was made. There was a suggestion that severe epidemics occurred at intervals of four years, that is to say two mild or clear years might follow a severe epidemic. This condition of affairs was particularly noticeable within recent years."
Parthasarathy & Sundararajan (1937)	Found that the periodicity of cholera in Mysore State in India "is most likely to be of six years duration."
Sen (1948)	Recorded in a study of the vital statistics in the United Provinces (now Uttar Pradesh) that "As regards cholera, there was uniformly a 3-year epidemic cycle till 1930 and thereafter the pattern seems to have changed. The years 1930 1938 and 1945 were high epidemic years for the province."
Benjamin (1949)	Stated in a report on cholera in Bombay State that "Considering the province as a whole, widespread severe epidemics, i.e. involving more than half the districts of the province, have occurred roughly at intervals of 3 to 4 years during the first two decades after 1901 and at irregular and longer intervals since 1921"
Duggal (1949)	Found that in Bihar the peak of moderate epidemics was reached at an interval of about five years and that of severe outbreaks after 12 to 14 years.
Banerjea (1951)	Maintained that "Seeing the mortality rates over a long period of 1877 to 1948 one may say that cholera in U P [United Provinces] occurs at irregular intervals and is largely influenced in its periodic explosiveness by big fairs and festivals held from time to time in the province. The three years periodic cycle noticed by other workers in this province may be due to the occurrence of Kumbh and Ardh Kumbh [i.e., extraordinarily frequented] fairs at Hardwar and Allahabad which recur after every three years."

of the epidemic index it was possible to forecast epidemics two or three months ahead of their actual occurrence They maintained, therefore, that

" By the use of the epidemic index graphs a watch can be kept for the possible out breaks of cholera and, as a corollary when an epidemic is forecast, preventive measures can be intensified when a calm period is indicated, energy and expenditure can be conserved."

It is important to note however that, as stated by Taylor (1941) the above method failed to give satisfactory results in areas with homogeneous cholera experiences. Taylor added that

" Another method based on the regression equation connecting cholera mortality of the last few weeks of a year with the total cholera mortality of the succeeding year gave more satisfactory results. This method gave good predictions for Calcutta for three successive years and was successful in 10 out of 19 of the homogeneous districts which have been designated for South-West Bengal."

Rogers (1928) claimed on the basis of his exhaustive investigations already referred to above that

" by watching the absolute humidity at seasons when it is commonly low enough to lessen the incidence of cholera, for any rise to over 0 400 favouring the recrudescence or spread of the disease, and taking into account the deficiency or otherwise of the previous rainfall, and the prevalence of cholera in surrounding areas, it should be possible in future to form a good idea of the relative danger of any pilgrimage, fair or other large gathering in any given place in time to issue warnings and to take other precautions to avert or lessen the impending danger "

As Rogers summarized in a lecture delivered in 1933 his method of fore casting cholera epidemics had given fully satisfactory results on repeated occasions Making a further study of the data available up to 1939 Rogers (1944) reiterated that

" 1 A close watch on the June to October South West monsoon rains enables high cholera incidence to be foreseen in the autumn months in the endemic areas with absolute humidities always over 0 400 and several months before the spread of epidemics of cholera in the next spring from the endemic to epidemic areas.

" 2. The danger of cholera spread by the return of pilgrims from any particular Fair can also be foreseen from the climatic data at the time and a knowledge that cholera is present in the areas through which the pilgrims have to travel."

Rogers s claims were not fully accepted by Taylor (1941), who reported that forecasts made according to the above method had proved successful in only 24 % of areas with homogeneous cholera experiences However Napier (1946) was inclined to consider Rogers s method as the most successful as well as the simplest of the various forecasting procedures.

Role of the serological races of V cholerae

Observations on the incidence of the serological races of *V cholerae* have been recorded in the following countries

Japan [illegible]

[illegible]

[illegible]

According to [illegible] (19[illegible]) [illegible] Hikojima type detected [illegible] in Japan

[illegible] that the successive waves of cholera epidemics in Japan were [illegible] the result of recent importations of the infection which thus never became entrenched in the country

Korea

According to Shiba & [illegible]shijima (192[illegible]) among [illegible]0 cholera strains examined by them those isolated during the 1921 outbreak in Korea showed the characteristics of the Inaba race

Manchuria

As established by Manako (1933) almost all of 187 cholera strains which had been isolated during the 1932 epidemic in Manchuria were of the Ogawa type

China

(a) Shanghai Writing in 1933 Nobechi maintained that as in Japan so also in Shanghai the Hikojima race of *V cholerae* was preponderant However Kuroya & Oho (1933) found that out of 53 strains isolated during the 1932 epidemic only five belonged to this race while 48 showed reactions corresponding to those of the Ogawa type According to Fournier (1939) the Hikojima type was once more preponderant in Shanghai in 1933 Nishimura (1938) found on the other hand that all 16 strains he had examined during the 1937 epidemic were Inaba strains

As stated by Fournier (1939) 93% of the 100 strains examined during the 1938 Shanghai outbreak were again of the Hikojima type However according to Fournier & Lieou (1943) the sporadic cholera manifestations at Shanghai in 1939 1940 and 1941 were caused by organisms of the Inaba race whereas all 65 strains examined during the 1942 epidemic showed the reactions of the Ogawa type

Japan

As noted in Chapter 4 Kabeshima (1913) examining 195 strains isolated during the 1912 outbreaks in Japan and Formosa found that the Japanese strains were of a "typical" serological character (or to use the nomenclature now in use were Inaba strains) whereas the Formosa strains were serologically "atypical", thus belonging to the Ogawa race of *V cholerae* Kabeshima's findings were soon confirmed by other Japanese observers who as aptly summarized by Venkatraman & Pandit (1938) believed

"that the original or Inaba type is associated with epidemic outbreaks and severity of infection and the varied or Ogawa type with sporadic cases and mild outbreaks."

According to Nobechi (1923 1933) from 1922 the "intermediary" or Hikojima type detected by him became predominant in Japan

Evaluating this change of type one must keep in mind that the successive waves of cholera epidemics in Japan were invariably the result of recent importations of the infection which thus never became entrenched in the country

Korea

According to Shiiba & Ushijima (1922) among 20 cholera strains examined by them those isolated during the 1921 outbreak in Korea showed the characteristics of the Inaba race

Manchuria

As established by Manako (1933) almost all of 187 cholera strains which had been isolated during the 1932 epidemic in Manchuria were of the Ogawa type

China

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The great variance of the above findings lends support to the view that the cholera manifestations in Shanghai were the result of repeated importations of the infection which thus never became permanently entrenched

(b) Yunnan Province Tang and colleagues (1944) typing 69 *V cholerae* strains isolated during the 1942 outbreak at Kunming, found 64 to be of the Inaba race while five, met with at the end of the outbreak were of the Ogawa type Tang and his co-workers considered it possible that this appearance of another serological race might stand in causal connexion with a reimportation of the infection from an area different from that responsible for the initial outbreak

(c) Szechwan Province According to Reimann (1947) out of seven strains which had been isolated during the 1945 cholera outbreak at Chungking, two were of the Inaba type, three of the Ogawa type and two of the Hikojima type.

Indochina

Genevray and co-workers (1939) recorded that they had found exclusively strains of the Inaba type during a limited but rather virulent cholera outbreak in a Tonking village

Burma

Out of 52 cholera strains examined by Maitra and colleagues (1938) at Rangoon 51 were of the Inaba type, and only one of the Ogawa type.

India

(a) Bengal As summarized by Taylor (1941) early investigations in Bengal had indicated the sole presence of cholera vibrios of the Inaba type but it was afterwards established that side by side with this race Ogawa strains could also be isolated. Further observations proving the co-existence of these two serological types may be tabulated thus

Author and locality	*Total number of strains*	*Inaba strains*	*Ogawa strains*	*Remarks*
Pasricha et al. (1939) Calcutta	379	266	113	
Read & Pandit (1941) Rural area	65	26	39	32 of these 65 strains had been isolated from patients, 23 from contacts and 14 from water samples.
Sen Gupta (1943) Calcutta	417	117	300	
Amberson (1945) Calcutta	159	5	154	

Author and locality	Total number of strains	Inaba strains	Ogawa strains	Remarks
Pasricha (1946) Calcutta	320	38	282	
Sen Gupta (1951) Calcutta	415 (from 200 patients)	112	302	One strain showed the serological properties of the *Hikojima* type
Gilmour (1952) Calcutta	190	65	122	In three patients of Gilmour s series the presence of a mixed infection with both Inaba and Ogawa vibrios was noted. In five further instances he recorded "*obvious reinfection* in the wards" the convalescents in question first excreting vibrios of one race (usually of the Ogawa type) and then, after a vibrio-negative interval, of the other type
Chakravarty (1954) Calcutta	1811	995	816	In agreement with the now generally accepted view Chakravarty found no marked differences in the mortality percentages of the patients succumbing to infections with organisms of the Inaba or Ogawa type.

It is important to note that, as shown by the observations of Sen Gupta (1943) covering the period from 1941 to 1943 during that time the incidence of the Inaba strains steadily declined the proportions being one Inaba type strain to 154 Ogawa strains in 1943 as against 71 to 76 in 1941 Then however the incidence of the Inaba strains again increased to become slightly preponderant in 1954

Another interesting statement made by Sen Gupta (1951) was as follows

"In a majority of the cases repeated examinations of stools were done and in most of such cases no change in sub-type was found in repeated isolations. But in 24 cases changes in sub-types were observed. Thus 8 cases showed a change from Inaba to Ogawa 10 cases showed a change from Ogawa to Inaba 4 cases showed a change from Inaba to Ogawa and back again to Inaba one case showed a change from Ogawa to Inaba and back again to Ogawa and one case showed a change from Inaba to Ogawa back to Inaba and again to Hikojima Of the 8 cases showing Inaba to Ogawa one showed the presence of both Inaba and Ogawa types in the same specimen so also did one of the 10 cases showing Ogawa to Inaba Of the four cases showing Inaba to Ogawa to Inaba one showed twice the presence of both Inaba and Ogawa types in the same specimen and one showed it once

Remarkable as these observations are one cannot share Sen Gupta's belief that he obtained proof of an *in vivo* transmutation of one subtype of *V cholerae* into another As has been emphasized in this connexion in Chapter 4 a transmutation of Inaba strains into Ogawa strains has not been effected even *in vitro* and appears to be improbable There can be little doubt, therefore that the simultaneous presence or successive appearance of the two subtypes of *V cholerae* observed by Sen Gupta in but 24 of his 200 patients was the result of mixed or successive infections with both kinds of organisms.

(b) Assam According to Taylor (1941) some strains of the Ogawa type had been isolated in Assam, but Inaba infections prevailed.

(c) Bihar As stated by Duggal (1949) before the year 1942 the Inaba type of cholera vibrios had been predominant in Bihar but since that time Ogawa strains had been found to predominate However in the Purnea district, Inaba strains were isolated—whether alone or side by side with Ogawa strains is not clear from the context.

(d) Punjab Examining 55 strains agglutinable with cholera-diagnostic serum, Yacob (1944) found that they were all of the Inaba type.

(e) Bombay Soman & Neil (1945) testing 164 strains which had been isolated during the 1943 and 1945 cholera epidemics in Bombay found only three Inaba strains as against 161 Ogawa strains

(f) Mysore Testing 63 cholera strains isolated during the 1949-50 outbreaks in Mysore State Rao and his co-workers (1952) confirmed the presence of 47 Inaba and 16 Ogawa strains

(g) Madras Dealing with early observations on the occurrence of Inaba and Ogawa strains in the Madras Presidency (now Madras State) Taylor made the following statement

"When a special investigation was carried out in the Madras Presidency in 1936 a series of 89 strains isolated in the previous years were re-examined and four of them were found to be Ogawa sub-type. In the same year it was found that strains isolated in Madras City and the Northern districts of the Madras Presidency were of Inaba sub-type, while the strains from areas in the Southern part of the Presidency were all of Ogawa sub-type. In one area in Madras [? Madura] district 84 strains isolated from cases were studied in detail [1] and all were found to agglutinate to 75 per cent to 100 per cent of titre with a pure Ogawa O serum and to 10 per cent only with Inaba O serum The observations made during the outbreak did not suggest that there was any difference in virulence or epidemiological features in the case of Ogawa sub-type infections as compared with the Inaba sub-type epidemics which are commoner in India.

"Investigations carried out over a period of years in the Madras Presidency have shown extensions and recessions of the areas in which the respective sub-types may be the forms associated with outbreaks. A complete change occurred, for example, in 1940 in which the Ogawa sub-type was the form associated with an outbreak in the Northern Circars, all 252 strains isolated in the area being of this type."

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[1] See Venkatraman & Panda (1938)

Summarizing the results of further observations on this point in the Madras areas, Pandit (1948) stated that during the period from 1939 to 1945 (when major epidemics took place in 1942 and 1943) Ogawa strains were almost exclusively present However after a year of low cholera incidence a change once more took place, all strains isolated in 1947 and 1948 being of the Inaba type

Commenting upon these and the previous findings in this region Pandit pointed out that

"This extension and recession of the area with regard to the prevalence of the types and a complete change-over from one to the other are matters of considerable epidemiological interest and may have a bearing on the cyclical periodicity of epidemic cholera in India."

Egypt

As stated by Gohar & Makkawi (1947) and several other observers the Inaba type of vibrio was exclusively met with during the 1947 cholera epidemic in Egypt

As mentioned already Taylor s above-quoted opinion that outbreaks caused by either the Inaba or the Ogawa type of *V cholerae* do not differ in seriousness or epidemiological features has now been generally accepted Nevertheless, systematic observations on the occurrence of these types are of considerable value, being likely to furnish clues in regard to the place of origin of cholera invasions in non-endemic areas or localities One cannot help noticing, however that with the exception of a few areas, no sufficient evidence is available for such inquiries

Causes of decline of epidemics

Since it is indicated to deal separately with the involved problem of the spread of cholera later in this chapter attention can now be devoted to the factors governing the decline and disappearance of epidemic manifestations of the disease

Dealing with the latter subject in his classical epidemiological study Flügge (1893) considered it puzzling that the cholera outbreaks did come to an end, even though the reported instances of infection involved only 3%-5% of the population and consequently many susceptible individuals remained present while the infection quantum ought to have become maximal after the outbreaks had lasted for some time

"This paradox" said Flügge, "is explainable in part by the fact that the season gradually becomes unfavourable for the spread of the epidemic in particular the individual susceptibility is lowered. Associated with this factor is the influence of the actual extent of the infection [*Durchseuchung*] This really involves a far larger part of the population than is indicated by the number of reported cases. We may assume that, in addition to the latter there are very many individuals with very slight infections which are not recorded but nevertheless confer immunity Finally it must also be borne

in mind that the people only gradually learn the proper prophylactic measures against cholera, the necessary care in regard to food and drink, the correct treatment of patients, etc. Those who are careless are, for the most part, attacked by the infection after a few weeks the careful individuals, who listen to good advice, have learned how to act, and thus there is a gradual falling off both in the careless dissemination of the causative organisms and in the number of susceptible persons." [Trans.]

It is interesting to compare this early statement with the following opinion given by Napier in 1946

" *The natural subsidence of an epidemic.*—This may be due to the favourable development of the climate, to some change in the water-supply or the availability of another from any cause, or to exhaustion of the clinical material. It has also been claimed that it is effected by the development of bacteriophage in the water supply "

As will be noted, both these observers, though in part making different postulations, were agreed that the subsidence of cholera epidemics was the result not of one factor but of a combination of various factors. These will now be considered seriatim.

Climatic factors

Since, as has been discussed above, a high temperature and a suitably high atmospheric humidity promote the epidemic spread of cholera, it is clear that the outbreaks are bound to subside *pari passu* with the appearance of increasingly temperate climatic conditions The cooler the weather becomes the less the people will be inclined to consume unsafe cold drinks and foods which can be easily contaminated with *V cholerae* There will be also a decrease of flies which play a most important role in producing such contamination as well as in conveying gastro-intestinal affections due to other micro-organisms which, as discussed before, in their turn are apt to lower the resistance to cholera infection

An increasing dryness of the atmosphere is apt to exert an adverse influence on the survival of the cholera vibrios on foodstuffs like vegetables and fruits, which play a dangerous role in the spread of the infection

Recognition and abolition of sources of infection

As will be fully discussed in the following chapter indispensable pre requisites for an efficient control of cholera epidemics are (1) a fully adequate intelligence service having at its disposal a well-equipped and well-staffed laboratory branch to ensure prompt investigation of suspicious patients and the rapid establishment of the diagnosis of cholera (2) sufficient facilities for isolating the patients so as to cut short the spread of infection by them (3) a sanitary engineering service to deal with, or if necessary to prevent the further use of contaminated water supplies furnishing safe water in their place (4) implementation of measures to control or if necessary to prohibit the sale of potentially dangerous cold drinks and foods and, hand in hand with this adoption of the necessary methods of fly

control (5) large scale public health propaganda to ensure that the people themselves take all necessary precautionary measures

Carrying out this complicated programme in unforeseen or unexpectedly extensive cholera outbreaks is fraught with great initial difficulties However, *pari passu* with the creation of adequate facilities for such work the out breaks are bound to subside It is obvious that the results of such programmes will be most satisfactory if the period of their maximum efficiency coincides in time with climatic conditions unsuitable for the spread of cholera

Loss of virulence

As already alluded to in Chapter 6 there can be little doubt that the drop in mortality figures often though by no means invariably observed during the later phases of cholera epidemics is not the result of a hypothetical change of virulence but is due to extrinsic causes owing to a gradual improvement of the case-reporting system more slightly affected patients are detected while for the same reason those attacked with cholera gravis are more promptly hospitalized and thus become more amenable to treatment This view has already been advocated by Nichols & Andrews (1908) who stated that during the major cholera outbreak taking place at Manila from August to October 1908,

> "The mortality in the last part of the epidemic was only 5 per cent less than that of the first part. This decrease may be much more reasonably attributed to increased facilities for finding and treating cases than to reduced virulence "

Acquired immunity

Observations speaking in favour of the now widely accepted postulation that cholera outbreaks lead to the production of a herd immunity in the populations concerned had been made long before the discovery of the *V cholerae* Thus Koch (1885) referred to early experiences in India which had taught that recently arrived European troops suffered much more severely from cholera than the native regiments Analogously it was found advantageous to employ Indian nurses in the cholera wards of the military hospitals because they were not likely to contract the infection Koch further stated that during the Crimean War and the Austro-Prussian War of 1866 it had been noted that the arrival of new troops in places where cholera was on the wane gave new impetus to the infection Taking account of such observations the *Cholera Regulativ* (cholera regulations) by Griesinger Pettenkofer & Wunderlich (1866) stated that if

> "a body of troops has experienced cholera it acquires in this manner for some time a certain degree of insusceptibility or immunity against the infection." [Trans.]

In Koch's own opinion widespread cholera outbreaks in India engendered a herd immunity lasting for three or four years then the infection

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the ideas of the two long-established schools of epidemiologists—the "contagionists" and the "localists" The former insisted that cholera was a highly contagious disease to be guarded against by a rigid system of measures consisting of isolation of the patients as well as of elaborate methods of disinfection and quarantine The "localists", on the other hand maintained that the manifestation of cholera as well as of other infectious diseases was dependent upon local "miasmatic" factors and to the delight of those interested in traffic and commerce, considered all the above-mentioned control measures unnecessary As Flügge—from whose masterly publication the statements just quoted have been culled—noted the constant tug-of war between the two schools is well shown by the following quotation from a 1831 summary by Lichtenstädt

"The subject of contagion has been much debated. The non-contagionists have found many adherents among the medical men and gained the support of the public, which sees in quarantine work only what is irksome and disadvantageous. Though one must unhesitatingly admit that the system of cleansing measures led to exaggerations, an unbiased observer cannot consider this as great an evil as the possibility of a spread of this disease May there be finally an end to the often reiterated postulation of the non-infectivity of cholera! It belongs in the category of the most dangerous errors of our time." [Trans.]

As summarized by Flügge, Pettenkofer—who became the protagonist of the "localists" through a voluminous series of publications which began to appear in 1855—conceived the following hypothesis

The still unknown causative organism of cholera (x) was spread through human traffic not only by patients but also by healthy persons. However in order to become capable of causing infection (*infectionstüchtig*) it had to reach a soil possessing suitable characteristics (y) the favourable condition of the soil depending mainly upon a low ground-water level, which leads to some desiccation of the superficial layers.

The pathogenic agent resulting from the union of x and y (called z) was supposed to produce air-borne infection in man. If the factor z was imported by patients or healthy people into a hitherto cholera free locality sporadic infections might result. However epidemics could develop only when the soil of the locality contained the y factor and when the time was ripe for an outbreak (*zeitliche Disposition*).

Though this hypothesis obviously became obsolete as soon as Koch had discovered the cholera vibrios and had proved that the faeces of the patients, since they teemed with the organisms in a virulent form, served as the immediate vehicle of the infection, Pettenkofer and his school tenaciously adhered to their ideas However their attempts to adapt the facts to the Procrustean bed of their surmises were convincingly opposed by Koch at the 1885 cholera conference and Pettenkofer s views were also refuted by unbiased observers like Virchow (1885) and Liebermeister (1896) Nowadays Pettenkofer s theory is merely an historical curiosity even though his concept of an epidemic spread of cholera promoted by suitable local and seasonal influences remains basically sound

As is now generally accepted the local spread of cholera infection is governed by the following factors

spread once more from the endemic areas along the main route of traffic towards the north-west. Like Flügge (1893) he emphasized

"that in order to become immune, it is most likely not necessary to suffer from the disease in its most severe form but that a slight attack also confers protection against a second attack and so I assume that slight cholera attacks, even hardly noted cholerines, which are very frequent at the time of cholera, can also engender an immunity For this reason a considerably larger part of the population is to be considered as having passed through cholera attacks [*durchseucht*] than one could conclude from the reported case incidence or the mortality figures." [Trans.]

Sharing this opinion, Dunbar (1896) recorded that the examination of the stools of 111 contacts of a total of 15 cholera patients, met with in nine groups on two steamers and in seven houses, had proved positive for *V cholerae* in 28 instances Two of these 28 individuals afterwards developed cholera gravis, 11 having solid stools, were evidently carriers however 15 presumably had slight attacks of the disease, since they showed signs of diarrhoea.

Observations recorded by Barikine & Cazeneuve (1925) at Rostov-on-the Don showed that refugees were more susceptible to cholera infection than the resident population. The latter fell ill only after the infection had gained impetus among the former and the morbidity and mortality was higher in the refugee group than among the residents However one should not forget that the refugees, in addition to not being immune to cholera, were presumably also non-specifically less resistant to the infection, as a result of the vicissitudes they had endured during their flight and of con tinuing to live under unfavourable conditions.

Koch s belief that the herd immunity engendered by cholera outbreaks lasted for three to four years was not shared by other observers Some of them, like Basil (1910) believed that the state of immunity lasted longer Flu (1915) on the other hand, stated that it persisted for six months only while Heiser (1908) spoke in this connexion of a period of two years. It would seem likely that the length of the period is not uniform under all circumstances but depends upon the interaction of variable factors such as the duration and the extent of the outbreaks While an individual outbreak may fail to produce a solid herd immunity that immunity may eventually be engendered through repeated cholera manifestations [1]

Factors Governing the Local Spread of Cholera

Introductory remarks

As soon as the appearance of cholera in Europe facilitated scientific investigation of this disease attempts were made to classify it according to

[1] The influence exerted on cholera epidemics by bacteriophage action will be discussed in the following chapter

While contact infection does not seem to play a generally important role in the epidemic spread of cholera in India, it is of great importance for the perpetuation of the infection in sporadic form in the endemic foci (Napier 1946 1951)

Water-borne infection

Introductory remarks

As alluded to in the first part of this book, most convincing epidemiological evidence of the role of contaminated water in the spread of cholera was furnished by Snow (1855) His inquiries left no room for doubt that a most violent local outburst of the disease in 1854 during which in the immediate vicinity "of the spot where Cambridge Street joins Broad Street there were upwards of five hundred fatal attacks of cholera in 10 days" stood in causal connexion with the consumption of the water from the Broad Street pump Equally illuminating was the difference of cholera incidence in the houses supplied respectively by the Southwark and Vauxhall Company obtaining their water from a polluted part of the Thames river and by the Lambeth Company which had recently removed their intake to Thames Ditton "thus obtaining a supply of water quite free from the sewage of London" The figures in question were thus tabulated by Snow

Water supplied by	*Number of houses*	*Deaths from cholera*	*Deaths in each 10 000 houses*
Southwark and Vauxhall Company	40 046	1 263	315
Lambeth Company	26 107	98	37
Rest of London	256 423	1 422	59

Thus, Snow commented

"the mortality in the houses supplied by the Southwark and Vauxhall Company was therefore between eight and nine times as great as in the houses supplied by the Lambeth Company and it will be remarked that the customers of the Lambeth Company continued to enjoy an immunity from cholera greater than the rest of London which is not mixed up as they are with houses supplied by the Southwark and Vauxhall Company"

In contrast to the opinion usually held that polluted water merely favoured cholera infection "by predisposing or preparing the system to be acted on by some unknown cause of the disease existing in the atmosphere or elsewhere" Snow asserted

"that, if the effect of contaminated water be admitted, it must lead to the conclusion that it acts by containing the true and specific cause of the malady"

It was presumably due to the influence of Pettenkofer s theories that the clear-cut evidence of Snow was not or at least not fully accepted. Thus Griesinger (1857) while admitting the possibility of water borne infection, considered it to be exceptional A subsequent claim by Farr (1866) that defects in the filter plant of the East London waterworks, which

Contact infection

Though invariably contracted in the immediate vicinity of cholera patients infection by "contact" may be effected in various ways The causative organisms may be introduced into the mouth of those near the sufferers by fingers soiled with the faeces of the latter particularly if those attending the patients partake of meals without having properly washed or *disinfected their hands But in other instances the infection is not quite so* direct, things like eating-utensils or foodstuffs, which had become contaminated with *V cholerae* while kept in the vicinity of the patients, serving as vehicles of the infection

Assessing the importance of contact infection in the spread of cholera, one must first of all fully agree with the statement of Koch (1893) that this disease is by no means as highly contagious as, for instance smallpox and measles "in which simple contact or even a passing sojourn in the sickroom suffices to produce infection"

This difference is easily explainable when it is considered that oral introduction of the causative organisms is the only means of effecting infection

Opinions of different authors regarding the frequency with which contact infection as defined above takes place in cholera outbreaks vary widely Some consider it a frequent or even the common means of the spread of epidemics, while others assert the rarity of this mode of infection, pointing out in particular that the disease does not usually show a tendency to familial spread, infection in one inhabitant of a house as a rule remaining confined to that person

Scrutinizing this discrepant evidence more closely one can easily perceive that these marked differences depend upon the conditions under which the population groups concerned live It is true that, generally speaking, cholera shows no tendency to spread in ordinary households However contact infection may become rampant in premises where people live crowded together under particularly insanitary conditions, as, for instance, in camps for pilgrims or seasonal labourers or in badly managed billets for soldiers This state of affairs was recognized at an early date by Snow (1855) who spoke of the "communication" of cholera

"in the crowded habitations of the poor in coal-mines and other places, by the hands getting soiled with the evacuations of the patients, and by small quantities of these evacuations being swallowed with the food, as paint is swallowed by house painters of uncleanly habits, who contract lead-colic in this way"

The cholera epidemics arising through contact under such particularly unfavourable living conditions may be of an explosive nature Generally speaking, however the curve of contact epidemics as distinct from major water borne outbreaks, tends to be flat the infection creeping from one group of people to another (Koch 1893)

Kinds of water supply involved

(1) Waterworks water Though, as described above a strong case for a role of waterworks water in the spread of cholera had already been made through some early observations, particularly those of Snow and Farr there is much reason to consider the 1892 cholera outbreak at Hamburg as the classical example of this mode of infection

As can be gathered from an excellent summary by Kolle (1904) Hamburg at that time quite unbelievably possessed no facilities for filtering the water of the river Elbe from which the city waterworks drew their supplies Thus with the aid of an intake the unfiltered river water was led into conduits (*Kanäle*) and then pumped into the pipe system. True the intake was situated above the city but, owing to the tides it was regularly reached by waves from the port. How the initial infection of the latter took place could not be definitely established but a role of Russian emigrants, who had come from cholera areas existing in the east and had been housed in sheds near the port was suspected with much reason

For several weeks after the commencement of the outbreak only sporadic cases, involving almost exclusively the port area, were recorded However on 20 August when no doubt the infection had reached the pipe system an explosive spread of cholera commenced the number of daily admissions reaching about one thousand by the end of the month. As stated in the first chapter the total number of patients recorded during the outbreak was 19 891, with 7582 deaths at the same time, as shown by a tabulation in that chapter, the two adjacent communities of Altona and Wandsbeck, which derived their water supplies from other sources, suffered markedly less Thus, as justly emphasized by Kolle, these observations furnished convincing proof of Koch s postulation that the spread of cholera was mainly due to contaminated water supplies and at the same time showed the untenability of Pettenkofer s theory Kolle stated in this connexion the following

"As is known, the political borderline between Hamburg and Altona is actually completely inapparent. It exists only on the map the transition from one of the cities to the other is so imperceptible that usually one cannot recognize the borderline, for instance, whether in a given street one is in the territory of Hamburg or Altona. In the maps on which all cholera cases had been shown, one found—not surprisingly for the adherent of Koch s theory—that the distribution of the cholera cases coincided strictly with the territory of the water supply and was limited within the political border which also formed that of the water supply system. It was observed that on one side of a street numerous cholera cases occurred, whereas the other side remained completely free. Both sides of the street were on the same ground, had the same subsoil, the same sewage system over the street was the same heaven, shone the same sun and nevertheless one side remained free from cholera, whereas on the other side numerous cholera cases occurred. Thus the houses and the inhabitants of this street had everything in common except one thing—the water supply system." [Trans.]

Further noteworthy observations on the role of water derived from faultily functioning filter plants of waterworks in the causation of cholera

had become grossly manifest by the presence of small fish in the tap-water were responsible for a cholera outbreak was also received with quite unwarranted scepticism.

No notice seems to have been taken either of the following interesting observations recorded afterwards by Dehio (1892) In Reval (Russia) there was a cholera outbreak in 1871 which terminated on 21 November In the course of December the cesspools of the city were voided and their contents were deposited outside the city on snow-covered ground, through which ran a canal servicing a city waterworks. Warm weather setting in at Christmas led to an inflow of the melted snow into the canal and cholera immediately became recrudescent in Reval, remaining, however restricted to the precincts in which water from the canal was utilized

However irrefutable proof of the occurrence of *V cholerae* in the water supplies used for human consumption and of the epidemiological importance of such contamination was furnished through observations of Koch, who reported thus on his initial findings in India at the 1884 cholera conference

"I succeeded in finding the comma bacilli with all their characteristic properties in a tank which supplies water for drinking and household purposes for all persons living round it and in the immediate vicinity of which a number of fatal cholera cases had occurred. As was established later the linen of the first cholera victim succumbing nearby had been washed in the tank On its shore there were 30-40 huts inhabited by about 200-300 persons and 17 of these had died of cholera. How many had been ill with cholera could not be established with certainty " [Trans.]

As further stated by Koch, a corollary to these observations was that the opening of the Calcutta waterworks in 1870 had been followed by a two-thirds reduction of the cholera incidence in the city while the incidence of the disease in the suburbs remained unaltered. That the city did not become quite free from cholera was due to the fact that a considerable part of the population continued to use river or tank water Fort Williams could be kept entirely free from cholera through the exclusive use of tap-water Similarly Pondicherry ceased to suffer from the infection after the installation of artesian wells

Koch's findings were confirmed by a number of other early observers (see summaries by Kolle 1904 and by Greig, 1929) One must agree with the statement of Greig that owing to the unavailability of serological methods these early observations cannot be considered fully authentic, but there is no reason to deny the validity of most of them, the less so because on numerous occasions the presence of *V cholerae* in water supplies has been confirmed by modern workers using exact methods of identifying the organisms. Referring to recent exhaustive investigations made in this respect in the course of a field enquiry in Bengal Taylor (1941) made the important statement that

"*V cholerae* was not isolated out of contact with the cholera case except on one occasion, although it was frequently isolated in the immediate vicinity of cases."

ment of the water is slowed down temporarily or permanently They [the vibrios] can then perish at the place where they have settled down or they may be torn off again by a stronger current Generally speaking, the unequal movement of the water in a pipe system must exert a considerable influence on the transport of the cholera bacteria and for this reason alone one pipeline may bring many and another few of the organisms to the corresponding houses. If the houses of the latter kind happen to be tenanted by well-to-do people whose habits of life render them little vulnerable to cholera infection, it may happen that whole rows of houses or even streets remain free from the disease." [Trans.]

Regardless of such inequalities of distribution cholera outbreaks caused by a contamination of central water supplies are invariably of an explosive nature their extent depending upon the size of the area supplied by the waterworks in question

(2) "Riverine" cholera An excellent explanation why "riverine" cholera, i e., infection contracted by the consumption of the raw water of rivers, plays a most ominous role in the spread of the infection not only in India but also in some other countries particularly in central and south China was given by Benjamin (1949) thus

"(*a*) Rivers their tributaries and even nullahs form the main, or often the only source of water supply on their bank.

"(*b*) Even where an alternative water supply e.g. wells, exists, there is a tendency on the part of the inhabitants to take the river water for drinking and domestic purposes, specially when the well water is slightly brackish or hard. [1]

"(*c*) In certain towns which are places of pilgrimage, even though a piped water supply is provided, there is a tendency for pilgrims to drink water direct from the river as the rivers are considered to be sacred

"(*d*) Gross pollution of rivers by utilisation of banks, or dry portions of beds of rivers for purpose of nature washing and bathing and washing of utensils in the river and what is more dangerous, the washing of infected clothes and utensils during cholera outbreaks entry of sullage from the town or village into the river and washing of dirt and filth from the town surface into the river by heavy showers. The continuous pollution of a river or stream, specially due to the washing of infected clothes and materials in it, tends to prolong the duration of the outbreak."

Earlier observations in Europe (see, for instance Flügge, 1893) as well as recent experiences in south China have shown that riverine cholera is particularly apt to affect people constantly dwelling on boats who because they have to rely almost invariably upon the consumption of the raw river water can easily contract the disease and whose faeces voided almost or quite directly into the rivers, form a most dangerous means of maintaining and spreading the infection Early and considerable cholera outbreaks among boat-dwelling populations in southern Chinese cities were therefore quite common (see for instance, Turnbull 1938)

As exemplified by the isolation of *V cholerae* from the water of the Yuan river (Hunan Province China) in the immediate vicinity of boats filled

[1] In the Szechwan Province of China the inhabitants of some communities indignantly refused the proposal to utilize, instead of the heavily polluted river water rain water collected in cisterns, because the latter, while running over the roofs, could become defiled by the excrements of birds!

outbreaks have been recorded by Koch (1893) at Nietleben in Germany (see below) at Astrakhan and in St. Petersburg by Kraus (1909) and at Shanghai, China, in 1926 (see Chapter 2) As noted below full bacteriological proof of the role of the water was obtained at Nietleben and the same was true of the epidemics at St. Petersburg and at Shanghai. No details are available in regard to the Astrakhan outbreak.

The epidemic in the asylum for the insane in Nietleben lasting from 14 January to 13 February 1893 and causing 122 cholera attacks with 52 deaths has been described in great detail by Koch (1893) How the infection was imported into the asylum could not be definitely established, but the fact that the disease quickly became apparent in different parts of the institution and also involved persons not receiving their food in the establishment attracted attention moreover there was no cholera in the clinical institutions of Halle receiving the same food supplies A water borne infection seemed probable therefore, and proof of its existence could be obtained. Evidently the faeces of the initial cholera patients had contaminated the sewage water which, owing to the cold weather prevailing, could not be disposed of properly by the irrigation fields As a consequence unpurified sewage found its way into the river water which also served as source of supply for the waterworks and which, as was found, was not properly filtered in the latter Soon after the cholera outbreak had commenced the presence of cholera vibrios could be demonstrated in the supposedly filtered water as well as in samples of the sewage water taken (*a*) before it reached the irrigation fields, (*b*) on the latter and (*c*) from the effluent.

As Koch commented with great reason, the uneven distribution of the cholera attacks caused by contaminated waterworks water which rendered many observers sceptical regarding the importance or even the existence of this mode of infection was conditioned by various factors It was well established that the individual susceptibility to cholera infection varied greatly Moreover Koch continued,

"the possibility of water-borne infection for different people was bound to be markedly different according to their relation to the water The one does not drink it at all and comes only into indirect contact with it owing to its use for household purposes and is, therefore, correspondingly less exposed to the danger of infection than another who drinks the water But even in regard to the latter it is of importance whether much or little water is drunk and at what time it is taken, when the stomach is empty or full whether the stomach and the intestines function properly whether excesses have been committed, etc." [Trans.]

On the other hand, Koch emphasized the distribution of the cholera vibrios was evidently not so uniform that each drop or each gulp of water contained the causative organisms Moreover even if the vibrios were at first evenly suspended, their distribution could afterwards become uneven For Koch said,

"One could easily imagine that they like other bacteria, occasionally adhere to solid objects, e.g., to the inside of a pipe system, which will be the case particularly if the move-

took place in a group of houses of Altona not provided with waterworks water so that a pump served as source of water supply A sewage system had been installed in the locality but this failed to function in the winter of 1893 when owing to the cold weather both the gullies and the ground round them became solidly frozen As a consequence waste water and sewage were bound to run into the inadequately protected pump shaft and it was obviously in this way that the water of the pump became contaminated with the dejecta of one of the few earlier cholera patients observed in the immediate vicinity of the pump An examination of the pump water at the end of the outbreak (31 January) revealed the abundant presence of cholera vibrios While specimens subsequently taken from the pump proved negative the organisms were found to persist for 18 days in one litre of the originally examined water kept in the laboratory at a temperature of 3° 5°C

The pump-water-caused epidemic at Sori near Genoa, Italy observed by Zirolia took place in the summer of 1911 when cholera was prevalent in the country Evidently the small river flowing through Sori became contaminated with *V cholerae* through the washing of the clothes of a cholera suspect patient and water from the river seeped through into an adjacent spring, which fed the pump serving almost exclusively at that time as a source of water supply for the town A total of 31 cholera attacks was recorded subsequent to the contamination of the pump water but only 27 occurring from 14 to 20 August, seem the direct result of this contamination, while the last four patients were apparently infected by contact Laboratory examination proved the presence of cholera vibrios in the water of the pump In a specimen of this bacteriologically positive water kept in the laboratory at a temperature of 16° 22 C the organisms were found to survive for 62 days without impairment of their agglutinability but showed a decrease in their virulence for intraperitoneally infected guinea pigs

(6) Springs As recorded by Lara (1927) an explosive cholera outbreak involving 25 patients and causing 23 deaths on the island of Romblon in the Philippines could be traced to the consumption of water from a spring found to have been contaminated with *V cholerae*—presumably through the dejecta of an earlier unidentified sufferer from the disease The level of this spring had become so low before the outbreak that owing to the disturbance of the bottom by the dipper its water was usually turbid This low water level must have led to a high concentration of the causative organisms, which no doubt accounts for the violence of the outburst *V cholerae* could also be demonstrated in two other springs, probably because they had been used for washing the clothing of cholera patients Since however the water of these two springs was not used for drinking purposes, their contamination does not seem to have proved dangerous for man.

with night-soil from a cholera infected city (Robertson & Pollitzer 1939) such transport which is usually made without any proper precautions, may also lead to a contamination of rivers

The character of cholera manifestations caused by a contamination of rivers varies considerably If they develop in a pilgrimage centre at a time when hosts of pilgrims bathe together in the rivers and simultaneously drink the sacred water explosive epidemics may result and—as will be discussed later—may lead to a wide spread of the infection Often, however continued contamination of the river water by the faeces of cholera patients may lead to the setting up of a vicious circle, the perpetuated interchange of the causative organisms to and from the water resulting in much less violent but very protracted outbreaks

(3) Irrigation channels In some areas irrigation channels play a dangerous role in the spread of cholera. This is particularly true of a large part of Madras Province (Madras State) where according to Mathew (1949)

" smaller irrigation channels pass through every village in the area. Almost each house has direct access to the channel. The channel water is grossly contaminated by personal ablution, washing of clothes and vessels, washing of animals, etc. Even the clothing and bedding soiled with cholera excreta are washed in them and yet the people drink this water as such as they find it to be more tasty than well water "

(4) Tanks and ponds The early claim made by Koch (1884) regarding the dangerous role played by village tanks (ponds) in the spread of cholera, recorded at a time when no fully reliable methods were available for the identification of *V cholerae* has been fully supported through adequate modern investigations in both the endemic and the non-endemic areas of India. Discussing the role of these sources of drinking water supply in the non-irrigated areas of Madras State Mathew (1949) recorded that the ponds and tanks

" are seldom properly protected or conserved. Their water level will be very low during the period March to June, and many of them may even dry up This is also the festival season and the period of maximum atmospheric temperature. Any cholera infection of the water source at that time will give rise to an explosive outbreak of the epidemic as the dose of infection is more concentrated."

It would seem, however that explosive outbreaks of this nature are exceptional rather than the rule Usually a repeated contamination of tanks or ponds by means identical with those responsible for cholera infection of rivers and irrigation channels tends to lead to protracted outbreaks

(5) Pumps Observations in the role of the water of pumps (*Brunnen*) in the causation of cholera outbreaks were made after discovery of *V cholerae* by Koch (1893) and by Zirolia (1913) who recorded the following interesting findings

The outbreak described by Koch (1893) which occurring towards the end of January 1893 was responsible for nine attacks with seven deaths,

been made by Japanese observers As Takano and his colleagues stated in this connexion

"Cholera in Japan has always been thought to be due to the pollution of sea water by ships from abroad and by carriers and cholera patients landed in Japan. The improvements in preventive measures in recent years facilitate the early detection diagnosis, isolation and disinfection of cholera patients. Therefore, only occasionally do patients on land infect persons with whom they come in contact. The quarantine on land is almost complete. When cholera breaks out in a port it spreads along the coast, and it is very rare to see the disease carried into provinces far from the coast Thus cholera in Japan is spread directly or indirectly by polluted sea water"

General considerations

Origin of the cholera contamination of water supplies Attention will be drawn in the following section of this chapter to claims made to the effect that, if contamination of rivers or other water courses with *V cholerae* has taken place at one point, the organisms could be carried by the current to communities lying downstream from the localities originally affected and that in the communities thus reached water borne cholera outbreaks could follow As will be discussed, the evidence adduced in this respect cannot be considered fully convincing. Moreover even if such statements could be accepted at face value it is clear that such "primary" contamination of the water supplies in individual localities could occur only because previously in another locality of the area in question there had been an invasion of the water supplies by cholera vibrios derived from human sources That the presence of cholera in man is thus an indispensable prerequisite for the contamination of water supplies, which then secondarily become the vehicle of the infection has been proved by many observations showing that the water samples invariably yielded positive results for *V cholerae* only after the disease had become manifest in man Results of large-scale studies proving this point have been recorded for instance by Dunn (1929) and Read & Pandit (1941) in India, and similar findings were made by the present writer in the course of a prolonged investigation of the vibrio fauna of the Shanghai surface waters. Dealing with this problem in a general manner, Greig (1929) quoted the following interesting statement by Houston (1913)

"If the immediate cause of what are recognized as water epidemics in the past could be precisely ascertained, I believe in most cases it would be found that accidental infection of the supply by what is known as a porter or carrier of disease had occurred."

In Greig s own opinion

"The human reservoir is in a position to supply an adequate dose of poison to various distributing channels—water milk, flies, etc., and so initiate epidemics of cholera. It will be seen that the problem of the prevention of cholera is, shortly stated, the protection of mankind from man."

(7) Wells As shown by observations in several cholera affected areas, particularly in parts of India (e g. in Bombay State) and in south and central China, wells may for various reasons, play a dangerous role in the local dissemination of the infection. If the wells are not or inadequately protected or faultily constructed in general, they may be contaminated with materials containing *V cholerae* which had been deposited nearby and may either be washed into the mouth of the well from the surface or may reach the interior of the well through seepage such entries of the polluted materials being particularly likely to occur during showers Faulty methods of collecting the water may render even properly installed wells dangerous Thus in China the wells were as a rule not provided with permanent fixtures to draw their water so that the people had to bring buckets and ropes of their own which, since they were ordinarily kept in the houses in a rather careless manner could quite easily become contaminated during outbreaks with the dejecta of cholera patients or otherwise come in touch with materials containing the causative organisms Repeated contaminations of the well water could thus be effected if cholera became manifest successively in various households obtaining their water supplies from the wells in question.

While shallow wells are generally involved in the local dissemination of cholera rather than the deep wells which are almost invariably far more solidly constructed, this rule is not without exceptions. For instance as recorded by Sian (1931) during an outbreak taking place in 1930 in a badly sanitated area in the Philippines, three out of 10 artesian wells examined for the presence of *V cholerae* yielded positive results.

Successive contamination of the often numerous wells used in cholera affected localities may contribute to the occurrence of outbreaks of a protracted type At the same time, however the manifestations due to the consumption of water from individual cholera-contaminated wells, though as a rule limited in extent, may be of an explosive nature The occurrence of such localized outbursts is well illustrated by an observation recorded by Takano and colleagues (1926) according to which consumption of the water of a cholera-contaminated well led to the infection of five individuals within less than 24 hours.

(8) Water supplies on ships As exemplified by observations of Brau (1905) and of Defressine and co-workers (1912) cholera contaminations of the water supplies stored on board ship may be the cause of outbreaks of the disease among the crews of the vessels In marked contrast to these experiences, the cholera manifestations observed during the 1892 epidemic aboard two ships moored in Hamburg appeared to be due to the consumption of water currently taken from the Elbe river (see Koch, 1893)

(9) Sea water Claims that cholera-contaminated sea water plays a most dangerous role in the causation of outbreaks of the disease have

"The persistence of cholera in a district is indicative of more than a single pollution of the water supply and generally points to a persistence of some insanitary conditions which favour repeated infection"

Similarly Robertson & Pollitzer (1939) judging from ample observations on water borne cholera outbreaks in China, came to the conclusion that

"Though there can be no doubt that river water contaminated with cholera vibrios formed the most important vehicle of infection, we are far from asserting that it served as a permanent reservoir of cholera. We believe that a kind of vicious circle existed infected faeces contaminated the river water which in its turn produced human cases."

It is possible however that the persistence of the cholera vibrios in contaminated water supplies is of some importance in helping to carry over the infection in the endemic areas

Role of contaminated food and drink

General considerations

Food and drink destined for human consumption may become dangerous vehicles of cholera infection in various ways most important among which are the following

(*a*) A dangerous practice adopted in some countries is to use fresh night soil as manure for lettuce and other vegetables destined to be consumed raw or after inadequate preparation, such as short pickling. As maintained by Takano and colleagues (1926) for instance the infection may be spread by this means at the time of cholera epidemics in Japan

(*b*) Foods like salads and jellies, to be consumed cold, as well as lemon ades or other cold drinks, may become dangerous if water polluted with *V cholerae* is used in their preparation It is obvious that such polluted water is likewise apt to prove dangerous if it is used for cleaning articles like vegetables and fruits to be consumed without cooking.

(*c*) It has also been claimed that the handling of foodstuffs by cholera patients may lead to a spread of the infection. Thus Seligmann (1918) maintained that a small cholera outbreak at Berlin in 1918 ascribed to the consumption of raw or improperly cooked horsemeat by most of those affected stood in causal connexion with the handling of the meat by a butcher who on the following day succumbed under circumstances suspi cious of cholera. It is also noteworthy that Eugling (1938) claimed to have observed cholera infections among soldiers who acting against orders had eaten uncooked corned beef from open tins they had found in the possession of cholera victims However the validity of these observations especially that of Eugling, is doubtful, and it is certain that instances of infections produced in the manner described by him or by Seligmann, if they really occur must be rare

(*d*) Cholera contamination of food or drink to be consumed cold may occur while the articles in question are stored or offered for sale. They may

Mechanism of infection. Discussing the means by which cholera-contaminated water was apt to produce the disease in man, Flügge (1893) made the following important statement [1]

"The infection by means of water which contains comma bacilli can be due to its mere use for cleaning the eating and drinking utensils, rinsing the beer glasses, etc. Certainly by far the most frequently it is produced through *drinking* of the water in question. The more the heat of the summer increases the urge to drink water the more frequently does this mode of infection come into question. It is all the more dangerous because the comma bacilli in a drink of cold water probably pass the stomach most easily without becoming damaged. If water is introduced into the stomach a small amount immediately passes into the small intestine after about an hour there is a rapid passage of the remainder but even this does not show an acid reaction, so that the comma bacilli have had no damaging influence and could enter the small intestine unharmed. Hence water is the substrate in which the comma bacilli are best preserved and in which they can reach the small intestine most easily The numerous epidemiological observations, the repeated demonstration of the comma bacilli in water and finally the special ability of water to lead the bacilli without harm through the stomach, render it quite certain that drinking water plays a *prominent* role in the spread of cholera." [Trans.]

Persistence of V cholerae *in contaminated waters* Claims that cholera vibrios may survive for prolonged periods in contaminated waters have been made by some workers Thus Defressine & Cazeneuve (1913) maintained that (*a*) running waters may remain infected with *V cholerae* for one month without reinfection (*b*) the organisms were able to survive in the mud and slime of the river bed in Toulon, France for as long as six months, and (*c*) this prolonged persistence of the cholera vibrios in surface-waters was of great epidemiological importance It is not possible, however to place much reliance upon these findings, made at a time when no reliable methods were available for a differentiation of the true cholera vibrios from cholera like water vibrios In the experience of most workers, particularly during warm seasons when cholera epidemics usually occur the period of survival of *V cholerae* in natural sources of water supply has appeared to be much shorter than the two French workers assumed. *Read & Pandit* (1941) who examined numerous water samples collected from various sources in areas of Bengal where cholera was present, noted in this connexion that only about half (9) of 17 initially cholera positive specimens showed evidence of the persistence of the organisms for more than five days and that the maximum period of their survival did not exceed 16 days.

As far as the present writer feels entitled to judge however he is led to believe that the question of the length of survival of *V cholerae* in natural sources of water supply possesses less practical importance than is sometimes assumed, because at the time of an epidemic prevalence of cholera the role of the contaminated water supplies appears to depend upon their repeated reinfection rather than upon a single invasion of the causative organisms. This idea seems to have been advocated at an early date by Hart and colleagues (1910) who stated that

[1] Compare a similar statement by Koch (1893), quoted above.

Dönitz mentioned the possibility that people in Tokyo coming in touch with the diseased fishermen might have contracted the infection by contact but there can be little doubt that the contamination of the fish played a preponderant if not an exclusive role because outbreaks due to the latter cause continued to be common in Japan (see Kabeshima 1918 and Takano and co-authors 1926 quoted in Chapter 6) In fact one of these epidemics, which took place in 1922 and was described in the 1937 report of the Eastern Bureau of the League of Nations Health Organisation had exactly the same history as the outbreaks observed by Dönitz

"There had been some severe cases of gastro-enteritis in a sea port about sixty miles from Tokio and, before a diagnosis of cholera was made, cargoes of fish had been sent to the Tokio fish market and distributed to various fish merchants. As a result, cholera cases were suddenly reported from all parts of the city to the number of nearly thirty cases a day for the short period the epidemic lasted. It was completely checked within two weeks by prohibiting fish from being brought into the city from the port in question"

Some circumstantial evidence incriminating oysters in the spread of cholera was recorded by Netter (1907) thus

"In the course of cholera epidemics, the disease on several occasions made its first appearance among persons who had consumed oysters.

"Thus in 1849 a cholera epidemic at Bridgewater and at Taunton became manifest among children who had eaten oysters which had been considered unwholesome

"In 1893 the towns of Grimsby and Cleethorpes near the mouth of the Humber had cholera cases. A fairly considerable number of persons affected in other parts of England had partaken of oysters from the beds in this locality either there or at a distance With each tide the oyster and mussel beds of Cleethorpes received materials carried by the sewers of both towns.

"Thorne reporting these facts in 1894 in the Local Government Report for that year expressed the opinion that the ingestion of oysters contaminated in these towns could have been responsible for these cases." [Trans.]

Heiser (1908) discussing the problems of cholera epidemiology in the Philippines drew attention to the particularly high prevalence of the disease there among the fishermen of Japanese nationality and also referred to observations which had shown that the early victims of the 1907 outbreak in northern Samar "all had eaten a poor quality of dried fish taken from waters in Manila that were presumably infected" He added the following curious statement

"The fishing industry in and about Manila is conducted on a most extensive scale The low marshy character of the section of the bay north of the Pasig River and the rise and fall of the tide produce ideal conditions for the growth and contamination of shell and other fish, and this is especially accentuated since the tidal currents are such that the contents of one of the city s largest sewers is extensively distributed throughout this area. Granting then, for the sake of argument, that fish, shellfish, and other sea products become infected with cholera and that through imperfect cooking the organisms are not all killed, and that one of the principal food substances of the masses in the city of Manila is gathered from the section just mentioned, it is evident that it would be possible to immunize a large portion of the city s inhabitants Enormous quantities of sea products in a dry or partially dry state are also sent to various portions of the

be put in containers which have become contaminated with *V. cholerae*—for instance, by having been cleaned with cholera polluted water—or they may become dangerous because they have been kept in the vicinity of cholera patients. A more common source of contamination is that articles like fruits or vegetables for sale are freshened up by the merchants or hawkers by sprinkling them with cholera polluted water. Another most dangerous practice is the frequent one of offering cold food or drinks for sale without protecting them against flies.[1]

As alluded to above, an adequate degree of moisture of foods like vegetables and fruits, which depends in its turn upon a sufficiently high atmospheric humidity, is an essential prerequisite for the survival of the cholera vibrios on these articles. This was clearly recognized by Flügge (1893) who stated that

"Foodstuffs, if kept in a moist condition, can long preserve the comma bacilli deposited on them by contact or through flies. . . . On many of these foodstuffs a multiplication of the comma bacilli occasionally seems to take place. However, the temperature, the degree of moisture of the substrates and the competition of saprophytes exert a very great influence in this respect, and usually little more seems to result than a preservation of the germs which, however, fully suffices for infection." [Trans.]

Fish and shellfish

The reasons for the important role played in the spread of cholera by fish and shellfish are that (*a*) in view of the frequent cholera pollution of the waters in which they live, they can easily become infected, or at least contaminated, with *V. cholerae*; (*b*) once the organisms have gained an entry, they are apt to survive for a considerable length of time or even to multiply; and (*c*) in certain countries, particularly in Japan and in the Philippines, raw fish form a staple product of the diet of the population, while shellfish, especially oysters, are consumed in the raw state on a fairly universal scale.

Early reference to the great epidemiological importance of fish in Japan was made by Dönitz (1886), who was able to establish that (*a*) during the 1877 cholera outbreaks involving Tokyo and its environs the disease raged with quite particular intensity in the coastal village of Haneda, through which most of the sea fish destined for the Tokyo market passed, and (*b*) cholera repeatedly became manifest en route among the fishermen bringing cargoes of fish by boat to the capital.

"It is unnecessary to state in detail," Dönitz remarked,

"how much the merchandise became defiled, since it would have been difficult for the boatmen to avoid contamination of the fish, even had they been fairly competent bacteriologists. Once the fish had become infected, they could spread the disease the more easily since in Japan the meat of some fish is sometimes consumed raw, being considered to be more tasty in this state." [Trans.]

[1] The role of flies in the spread of cholera, already dealt with in Chapter 6, will be further discussed in a later section of the present chapter.

In the course of the prolonged discussion of Pandit & Hora's hypothesis at the meetings of this expert committee, the necessity of further studying the possible role of hilsa fish in maintaining and causing cholera outbreaks was unanimously admitted However in the opinion of some of the speakers, part of the evidence adduced by Pandit & Hora in support of their thesis could be differently interpreted In particular it was pointed out that

" (i) the cholera endemicity map was not only similar to the hilsa fishery map but that there was also a remarkably close correspondence between the former and the maps showing the density of population (ii) the seasonal appearance of cholera in the inland focus situated along the Ganges in Bihar ascribed by Pandit & Hora to hilsa migrations, might be actually due to the influx of seasonal labourers."

Still more important than these objections is that systematic studies by Krishnan (1953) and by Pillay and co-workers (1954) furnished no convincing proof of the validity of Pandit & Hora s hypothesis

Krishnan failed to find cholera vibrios in 149 hilsa fish as well as in 317 common food fishes belonging to 32 other species caught in the Hooghly river at three points the water of two of which yielded *V cholerae* cultures upon several occasions Cholera like vibrios (which abounded in the river water) could be regularly isolated from the fish but were found to be less abundant in their gall bladder than in the intestinal and rectal contents When five of the fishes were kept in the laboratory in vibrio-free water which was changed daily excretion of cholera like vibrios was found to persist only for periods ranging from 2 to 14 days One of these fishes was found to excrete first cholera like vibrios for five days, then El Tor vibrios for one day and afterwards again cholera like vibrios However in view of the frequent occurrence of El Tor vibrios in India, it is not possible to share Krishnan s belief that their appearance in this fish was the result of a mutation and not of a mixed infection with both El Tor and cholera like vibrios

Summarizing the results of the hilsa fish inquiry conducted under the auspices of the Indian Council of Medical Research Pillay and colleagues stated that examination of (*a*) numerous specimens collected from the guts and gills of hilsa and other fish (*b*) many water samples collected from the surface and the bottom of the Hooghly river at the three above-mentioned points and (*c*) a fairly large number of samples of silt from the river had given the following results

Kind of specimen	*Results of bacteriological examination*	
	V cholerae	*Cholera-like vibrios*
849 specimens from 349 hilsa fish	Negative	Found in 136 specimens
669 specimens from 422 fish of other species	Negative	Found in 267 specimens
935 Hooghly water samples (see text above)	Found 10 times at 2 of the sampling points	Found in 437 samples
102 river silt samples	Negative	Found in 44 samples

Islands from Manila and if it should happen that some of them have been shipped in a moist condition or are otherwise rendered a good culture medium of cholera vibrio it is conceivable that such products might, in isolated instances, be the cause of appearances of cholera in places such as Samar Leyte, etc. In view of the fact that this class of food products is the sustenance of the masses because of its cheapness, it is more *probable that the underfed members of a community would be the first to be affected* by the slight infection it might contain."

Further observations on a role of fish and shellfish in the causation of cholera outbreaks in the Philippines have been recorded by Pottevin & Abt (1925) according to whom the consumption of small fish and crayfish was responsible for the appearance of the disease in 1916 and by Fuentes (1932) who referring to an epidemic at Barrio San Roque Lingig Surigao stated that raw sea foods (fish, crustaceans, algae etc.) served as important vehicles of the infection.

Dealing in the 1915 report to the Local Government Board in Great Britain with the subject of cholera Johnstone stated that the sun-dried fish largely consumed in the Noakhali district of Bengal, because it was most attractive to flies, evidently played a role in the spread of the infection

As may be conveniently discussed at the present juncture, some observers postulated that, besides serving as vehicles for *V cholerae* in the manner described above, fish played a still more important role in cholera by being apt to act as reservoirs of the infection This possibility seems to have been considered first by d Hérelle and colleagues who briefly stated in their 1930 memoir that

" It is probable that aquatic animals peculiar to Bengal and Indochina may be concerned, annelid, crustacean or mollusc, in the intestine of which the cholera vibrio can live for a very long time and more especially in the intestine of which the avirulent cholera vibrio can become regenerated."

In an elaborate thesis published in 1951 Pandit & Hora postulated that a role in maintaining cholera endemicity in India was probably played by the hilsa fish (*Hilsa ilisha*) because, as summarized in a report on their work embodied in the proceedings of the first session of the WHO Expert Committee on Cholera (1952)

" (*a*) A striking similarity was found to exist between maps drawn to show the main foci of cholera endemicity in India and those showing the areas where the main hilsa fisheries were located.

" (*b*) Some correlation seemed to exist between the movements of hilsa during the various seasons of the year and the seasonal variations in cholera incidence.

" (*c*) Apparently there was also some correlation between the five-yearly peaks in hilsa fishery and the periodicity of cholera in Eastern Bengal.

" (*d*) *The requirements suitable for a survival of the cholera vibrio in water namely* (i) a high organic content of the latter (ii) a suitable concentration of salts, and (iii) an absence of the lethal effects of sunrays, seemed to be fulfilled in the normal environment of hilsa which was migrating near the bottom of the rivers.

" (*e*) The methods of handling hilsa fish to prepare them for consumption are compatible with the assumption that these fish may play a role in the spread of cholera, the procedure being not dissimilar to the dispersal of the vibrios in the environment through faecal matter "

great importance to a spread of the infection through contaminated inanimate objects the so-called fomites During cholera epidemics they insisted therefore upon the rigid disinfection of all sorts of articles including many which are now considered quite innocuous Even the letters sent out from the affected localities were assiduously fumigated !

Dealing with the role of fomites in the spread of cholera at the 1884 cholera conference, Koch refuted the idea that letters and goods in general could serve as vehicles of the infection but quoted many observations showing that body linen and bedclothes polluted by the faeces of cholera patients played a dangerous role in this respect—an opinion which was shared by other early workers, for instance by Dönitz (1886) and by Flügge (1893)

Dönitz quoted in this connexion the following observation

"During the summer of 1885 a French warship came from Tongking to Japan and put in at Nagasaki. A few hours after the anchor had been dropped, an officer succumbed on board to cholera His linen was handed to a Japanese washerman, who fell ill with cholera and died His wife also fell a victim to the disease at almost the same time. At once further cholera attacks followed and in a few weeks an epidemic was fully developed" [Trans.]

Flügge (1893) referred to several other instances in which the contaminated linen of cholera patients apparently served as a vehicle of the infection He maintained in this connexion that if such substrates became subject to desiccation, the cholera vibrios soon perished but insisted that

"If the linen is closely rolled together so that a desiccation of the inner layers is impeded, and if such a bundle is kept at a low temperature in moist air e g., in a cellar one can still demonstrate live comma bacilli after 3-4 weeks, probably even after a longer time." [Trans.]

As pointed out by Flügge, other inanimate objects in the environment of cholera patients besides linen could also become contaminated, including various utensils carpets and the clothes of those attending the sufferers While cholera vibrios thus deposited on smooth surfaces succumbed within 24 hours owing to desiccation the organisms could survive for two days in porous cloth

The role of fomites in the spread of cholera was discussed at the international sanitary conference held at Dresden in 1893 when the conclusion was reached that, besides contaminated body and bed linen, only clothes and the wastage (*Abfälle*) of rags and cloth were apt to serve as vehicles of the infection (see Kobler 1913) However as will be discussed in the following chapter even this restricted list seems too long.

Role of flies

The results of laboratory investigations showing that flies play an important part in the spread of cholera have been confirmed by many epidemiological observations. The following among the latter deserve special discussion

The viability of cholera vibrios in the alimentary tract of artificially infected fish was studied by Pillay and co-workers with the following results

(*a*) "Specimens of the Climbing Perch (*Anabas testudineus*) and Murrel (*Ophicephalus punctatus*) which were kept under observation and had been found to have ceased to excrete NAG [i.e., cholera-like] vibrios, were infected by feeding them on artificially infected pupae and larvae of house-flies bred in the laboratory Each day the fish were removed to fresh sterilized aquaria and the water samples from the old aquaria were examined. But no vibrios were recovered for a period of more than 30 days. The fish were then dissected and their intestinal contents examined bacteriologically Haemolytic non-agglutinable vibrios were recovered."

(*b*) "In 4 sets of subsequent experiments the Climbing Perch, *Anabas testudineus* artificially infected with cholera vibrios, were found to excrete NAG vibrios of Heiberg's group II for a period ranging from 2-4 days."

That the cholera like vibrios isolated during these experiments were *V cholerae* which had lost their specific agglutinability—as Pillay and co-workers suggested—is difficult to believe because (*a*) those obtained at autopsy in the first experiment were in contrast to true cholera vibrios, haemolytic, and (*b*) those of the second experiment belonged to Heiberg's Group II and not to Group I, as true cholera vibrios almost invariably do

As will be gathered from the findings recorded above (1) Krishnan as well as Pillay and his colleagues never succeeded in isolating cholera vibrios from fish caught in the Hooghly river in the water of which *V cholerae* had been found upon several occasions at two of the three sampling stations (2) *V cholerae* showing the typical properties of this species and agglutinating with specific serum could not be isolated from artificially infected fish either It is noteworthy that Pillay and co-workers evaluating the observations they had made in the course of their investigations in regard to the cholera like vibrios, postulated

"that certain types of NAG vibrios may be mutant forms of the cholera vibrios and be responsible for maintaining cholera endemicity It can be conceived that cholera vibrios brought into the water from human sources, when consumed by certain fishes, mutate into NAGs and are excreted into the water in that form where they persist for long periods. These NAGs under certain conditions, as for example through rapid serial passage through the human intestines, may change back into true cholera vibrios and lead to cholera outbreaks."

However as has been stated in the fourth chapter it is impossible to believe in such a transmutation of "non agglutinable" cholera-like vibrios into true specifically agglutinable cholera vibrios It follows that thus far no convincing evidence has been obtained to support the belief that fish play a particularly important role in cholera epidemiology by acting as reservoirs of the infection.

Role of "fomites"

As alluded to in an earlier part of this chapter in the past the "contagionists" believing that cholera was a highly contagious disease ascribed

tuted by the practice of the hawkers of freshening up the fruits offered for sale with rags dipped in raw and often even dirty water

Referring to the subject at present under review in his valuable study on cholera in the United Provinces (now Uttar Pradesh) of India, Banerjea (1951) made the following statement

"In this province the worst months for cholera are those in which house flies abound, i.e summer and autumn months. Another thing which is observed is that while epidemics in rural areas are mostly explosive and localized in character those in towns and cities with protected water supplies but bad conservancy are protracted in nature with cases widely separated and are restricted to summer and autumn. These facts point to some connection which flies may have in the dissemination of the disease. According to Russell and Sundararajan (1928) the association of high relative humidity with high temperature accompanied with intermittent rains, forms the most favourable atmosphere for the development of the disease. All these, as pointed out by Ross (1928) are favourable conditions for the rapid multiplication of the flies. Heavy rainfall is unfavourable to the breeding of flies and it is noticed that a temporary decrease in cholera also occurs whenever there is a good rainfall."

Role of carriers

Early observations

The possibility that healthy carriers of *V cholerae* might play a role in the spread of cholera seems to have been considered long before the causative organism of this disease was detected Thus Griesinger made the following statement in 1857

"While the fact that patients suffering merely from diarrhoea can transmit and spread cholera has been indubitably established, it is not possible thus far to decide with full certainty that fully healthy persons coming from the place of an epidemic or generally speaking from a focus of the infection can bring the poison with them. Some observations render this most probable but there remains the possibility that such apparently healthy individuals had suffered to a slight degree at least from specific diarrhoea." [Trans.]

It will be noted that, while suspecting the existence of healthy carriers of *V cholerae* Griesinger also hinted at the possibility that persons who had recovered from a cholera attack might continue to harbour the contagion in their intestinal tract, thus becoming convalescent carriers.

That both these categories of carriers actually exist was confirmed fairly soon after the detection of *V cholerae* first apparently during the 1892 cholera epidemic at Hamburg by Dunbar (1896) whose interesting findings may be summarized as follows

Subjecting 142 stool specimens to smear examination, Dunbar obtained the following results

Category of specimens	*Number examined*	*Found positive*
Patients with manifest signs of cholera	68	41
Patients with diarrhoea only	47	25
Persons without clinical signs	27	4
	142	70

Flügge (1893) referring to the problem presently under review stated that

> "Various observers have shown that flies which have alighted on dejecta or on contaminated linen are capable of transmitting living comma bacilli even after some hours to articles of food. In small habitations without separation between the patient and the kitchen or the store-room, this mode of infection must be of serious importance in late summer and autumn, when masses of flies are present in such quarters." [Trans.]

It will be seen that Flügge was aware of two factors which facilitate the spread of cholera through flies—namely (1) a seasonal prevalence of these insects, which is apt to give impetus to the propagation of the infection and (2) nearness of the places where food is prepared or kept to the cholera patients or as observations in non-European countries have taught, to the latrines

Buchanan (1897) claimed that a cholera outbreak in an Indian jail was due to flies which had been carried by the wind from some nearby cholera affected huts into the prison compound The infection became manifest solely among those prisoners who partook of their food on the side of the jail near the affected huts.

Dealing with the importance of flies in the spread of cholera in the Philippine islands, Heiser (1908) stated that

> "Many isolated observations which seemed in some way to be intimately connected with the spread of the disease were made since 1903 It has been noted that when flies are particularly active and persistent and refuse to be driven away as for instance is the case in the United States before a thunderstorm immediately a considerable increase in the number of cholera cases almost invariably follows."

Attention to the role of flies in the spread of cholera in Java was drawn by Flu (1915) Making collections from 20 houses in which cholera patients had been found he was able to demonstrate the presence of *V cholerae* in the flies obtained from 10 of these dwellings. According to Tull (1920) flies were responsible for the spread of a cholera outbreak arising in 1920 in a labourers camp at Syriam in Burma. The insects were most abundant at the time and the cook house and the latrines were only 10 feet (3 m) apart.

Ample experiences convinced the present writer that in China as well flies played a prominent role in the spread of cholera. Outbreaks arising or running their course during the seasons of fly prevalence usually showed a wide spread and there was invariably a marked drop in the case incidence *pari passu* with the diminution of the flies. As noted by Robertson & Pollitzer (1939) the cholera situation always became particularly dangerous during the time when cut water melons were offered for sale on the streets or in open shops and the severity of the outbreaks abated as soon as the melon season had come to an end or the sale of the fruits was prohibited. No doubt cholera contamination by flies played an important role in rendering the cut melons dangerous. However an additional danger was consti

Moreover the possibility has to be seriously considered that the individuals in question instead of continuing to harbour cholera vibrios had actually become reinfected with these organisms Since, finally such long periods of harbourage have not been observed either by any of the modern workers or by most of those recording their experiences before 1935 it seems altogether unlikely that cholera carriers harbouring the causative organisms for periods longer than at most some months do exist

As can be gathered from a study of the voluminous literature on the subject, which has been ably summarized by Khan (1929) and by Couvy (1933) the views held by different authors regarding the importance of carriers in the spread of cholera varied most considerably Some maintained that even carriers of long standing played a dangerous role in this respect, while others reached the conclusion that the propagation of the infection depended solely upon what they called significantly, though somewhat incorrectly "acute" carriers i e., persons incubating cholera, patients in the actual phase of the disease and those in the early stage of convalescence. The relative merits of these contending views will be considered in the concluding part of the present disquisition

The statements made by the various authors regarding the frequency with which they had found healthy cholera carriers were also at great variance. Generally speaking since such carriers were mostly met with among persons in close contact with cholera patients, their incidence was bound to be highest in groups of people living crowded together in underprivileged households where no doubt unfavourable hygienic conditions favoured the direct or indirect passage of the cholera vibrios from the sufferers to their contacts Some authors also drew attention to the frequency with which small children were found to be carriers of *V cholerae*

Recent observations

Bringing up to date a report rendered by Pollitzer in 1952, essential recent observations on the carrier state in cholera may be tabulated thus

(a) Maximal periods of vibrio excretion in convalescent carriers

Author	*Number of cases*	*Maximal period of vibrio excretion (days)*
Tao Woo & Loh (1948)	?	9
Read & Pandit (1941)	1	13
Peterson (1946)	1	17
Gohar & Makkawi (1948)	1	23
Gilmour (1952)	1	25
Ying (1940)	1	21 28
Wilkinson (1943)	1	31
Kordi (1948)	1	33
Shousha (1948)	2	42

In this connexion Dunbar made the important observation that the faeces of the carriers contained fewer cholera vibrios than those of the patients. A corollary to this observation is that, as shown by the table inserted below the faeces of the carriers were not as a rule suitable for making a rapid diagnosis by cultural methods

Diagnosis established	*Patients with manifest cholera*	*Patients with diarrhoea only*	*Healthy carriers*
Within 15 hours	60 ⁄	40 ⁄	10 ⁄
Later	40 ⁄	60 ⁄	90 ⁄

Referring in a later part of his study to the frequency with which healthy carriers were met, Dunbar stated that during a recrudescence of the epidemic they had been found in "quite surprisingly" large numbers among immediate contacts of cholera patients In his opinion

"These findings furnish a new clue for the assumption that during cholera epidemics a far larger number of persons temporarily harbour the cholera vibrios than show manifest attacks of the disease. That this could lead to immunization cannot be excluded in the present stage of our knowledge." [Trans.]

As far as Dunbar could judge from a limited number of observations the causative organisms did not as a rule persist in the stools of cholera patients for longer than five days however in two instances faeces of convalescents continued to give positive results up to eight and 10 days respectively But the validity of these results was not confirmed by findings made by Simmonds (1892) when dissecting numerous victims of the 1892 Hamburg outbreak. For according to these observations *V cholerae* was still found to be present in more than half of the individuals who had succumbed on the seventh to twelfth day after onset of the disease though persistence later than that was exceptional, with a maximum of 18 days in a subject who after recovery from cholera had succumbed to pneumonia.

The pioneer observations of Dunbar were soon confirmed by some other workers, such as Metchnikoff (1893) Rumpel (1893 1894) and Abel & Claussen (1895) who seem to have been the first definitively to establish that excretion of cholera vibrios by carriers was apt to be intermittent rather than continuous.

Investigations up to 1935

The numerous observations on cholera carriers recorded during the present century up to 1935 cannot be accorded full credence, because it was only in the latter year that satisfactory methods for the identification of *V cholerae* became available Hence it is impossible to decide whether the earlier workers, especially those few recording a prolonged persistence of the causative organisms in the stools of carriers (for periods of a year or even a little longer) actually were in the presence of true cholera vibrios

(d) Incidence and average duration of vibrio excretion in contact carriers

Author	Number of persons examined	Percentage of carrier	Observations
Smith (1938)	10 407	2.84	Ships passengers arriving from the mainland in the Philippines
Read & Pandit (1941)	—	7 0	75 / free after 5 days
King Institute (1941)	61	6.56	Examined during a cholera outbreak
El-Ramli (1948)	2 035	4 1	Free after 5 days 50 / Free after 10 days 91 7 /
Kamal, Messih & Kolta (1948)	14 473	3 43	Intimate contacts of cholera patients usually became free from vibrios within 10 days
	2 411	1 9	" Stampeders " from cholera foci and boatmen examined at quarantine stations 13 of the 47 individuals harbouring *V cholerae* were afterwards found to be incubatory carriers
Kordi (1948)	2 037	4 1	Free after 5 days 50 / Free after 10 days 92.9 /
Shousha (1948)	13 702	2.1	Free after 5 days 65.96 / Free after 10 days 93.62 /
Wahid (1948)	600	2.66	Free after 2 days 75 / Free after 7 days 100 /
Hussein (1949)	2 027	4 14	Free after 5 days 50 / Free after 10 days 92.7 /
Venkatraman (1949)	245	2.04	These carriers were found among the staff members of a cholera hospital. In each of the five carriers *V cholerae* could be isolated but once
Gohar et al (1952)	1 745	3.56	Average duration of the carrier state was 4 4 days

From these data it emerges that the average duration of the carrier state has been found to be appreciably shorter in healthy or as many modern authors prefer to call them, contact carriers of *V cholerae* than in convalescent carriers While in at least 50% of the former the stools were found positive for not longer than five days and most of the individuals were free from vibrios after 10 days some observers continued to obtain positive results in a considerable minority of convalescent carriers during the second week following onset of the illness The maximal periods of excretion in contact carriers were definitely shorter than those found in the case of convalescents

(b) Average duration of vibrio excretion in convalescent carriers

Author	Number of cases	Observations
Ying (1940)	200	Positive up to 7 days 76.5% Positive up to 10 days 21.5%
Read & Pandit (1941)	10	Negative within 6 days 70% Negative within 8-13 days 30%
Peterson (1946)	1149	Average period of excretion 5.4±2.5 days
Reimann et al. (1946)	160	Excretion period usually not longer than 7 days
Tao, Woo & Loh (1948)	218	90% negative on or before 6th day none positive beyond 9 days
El-Ramli (1948)	689	Negative by 15th day 86.5% Negative by 20th day 93.6%
Kamal, Mesih & Kolta (1948)	1971	Positive not longer than 7 days 83.5%
Kordi (1948)	250	111 (44.4%) of these carriers were free from vibrios by the 7th day 208 by the 14th day
Shousha (1948)	463	Positive up to 10 days 56.16% Positive up to 20 days 29.80%
Hussein (1949)	250	Negative in 15 days 86.0% Negative in 20 days 99.6%
Cossery Ashouk & Hilmi (1949)	60	Majority negative in about a week's time, others within 4 weeks
Gohar et al. (1952)	78	96% became vibrio-free within at most 12 days, 4% within 20 days
Gilmour (1952)	113	71.6% were negative after the 1st week, 89.3% after 2 weeks and 98.1% after 3 weeks

(c) Maximal periods of vibrio excretion in healthy ("contact") carriers

Author	Maximal period of vibrio excretion (days)
Wahid (1948)	1
Read & Pandit (1941)	9
Gohar & Makkawi (1948)	10
Kordi (1948)	14
El-Ramli (1948)	15
Hussein (1949)	15
Omar (1947) (Quoted by Khalil, 1948a)	16
Shousha (1948)	19
Kamal, Mesih & Kolta (1948)	26

observations by Bruce White the results of which were thus summarized in the report on a meeting held in 1948 under the joint auspices of the Office International d Hygiène Publique and the World Health Organization

"At the end of the disease and during convalescence an increasing proportion of the vibrios excreted by the patient are in the process of roughening or are entirely rough. Transformation from the smooth to the rough state corresponds to a loss of pathogenicity of the organism."

As stated by Kamal (1951) these postulations were supported by findings made by Rainsford at the end of the 1947 epidemic in Egypt according to which cholera convalescents "excrete the R forms and none but the R' forms"

Since, however the observations of Gilmour (published in 1952) were not in agreement with these findings the experts taking part in the meetings of the WHO Expert Committee on Cholera (first report, 1952) came to the conclusion that

"so far no conclusive evidence was available as to whether or not and to what extent the vibrios excreted by convalescent and contact carriers tend to be rough and to have an altered virulence"

and added that

"It would be highly desirable to elucidate this point through systematic studies, advantage being taken of the recently recommended serological tests for the recognition of the R-type of *V cholerae*"

It follows that up to the present the problem of the role of carriers in the spread of cholera has to be elucidated with the aid of epidemiological rather than of laboratory observations.

Epidemiological observations on cholera carriers

In contrast to the now generally accepted views, some of the earlier observers believed that, as with other gastro-intestinal affections, like typhoid and dysentery in the case of cholera also carriers played an important role in the spread of the infection However the never convincingly documented claims made in this respect by Greig (1913a 1913b) and a few other writers were refuted in an exhaustive report on the role of carriers in cholera by Khan (1929) and in a series of further large-scale studies on this problem carried out under the auspices of the Office International d Hygiène Publique and published in 1933

The conclusion which Khan (1929) reached after a careful consideration of the experiences of previous workers and of observations on cholera convalescents and contacts he had made at Hardwar was that

"The reservoir of cholera is not the chronic carriers of *V cholerae* because they do not exist It is also not in the carriers of the inagglutinable vibrio because they do not cause epidemic cholera. The real reservoir is in the presence in the endemic areas of patients suffering or recovering from cholera. The only source of the infection

Confirming findings made in the past, some modern workers drew *attention to the frequency of the carrier state in infants or young children.* Abdou (1948) commenting upon these observations, postulated that the frequency of gastric achylia or of hypochlorhydria in children might be the cause of the high contact carrier rate in this age-group Be this as it may it deserves great attention that as shown by Gohar and colleagues (1952) achlorhydria or hypochlorhydria may be significantly frequent in adult healthy cholera carriers.

Infectivity of cholera carriers

Though some authors postulated that cholera carriers even those of long standing, were apt to play a dangerous role in the spread of the infection, many observers militated for various reasons against this view Some early workers, like Jatta (1912) and Piras (1913) stated in this connexion that the likelihood of a spread of the disease by healthy carriers was limited both because their dejecta contained fewer of the causative organisms than the stools of patients and because the stools of the carriers were solid instead of being diarrhoeic and, therefore much less easily diffusible. The markedly intermittent excretion of the causative organisms by cholera carriers no doubt also reduces the risks of spread of the infection

Attempts have been made by a few workers to test the virulence of the cholera vibrios excreted by carriers with the aid of guinea pig experiments. While observers like Ravenna (1911 12) Pontano (1912) Pane (1912) and *apparently also Babes (1914) found no appreciable difference between the* strains of cholera patients and carriers tested in this manner van Loghem (1911) recorded that the *V cholerae* cultures he had isolated from two healthy carriers proved to possess little virulence. Van Loghem pointed out, however that these observations on guinea pigs did not necessarily imply that the organisms in question were also innocuous for man.

A large scale attempt to test the virulence of cholera strains obtained from both patients and healthy carriers with the aid of guinea pig experiments (intraperitoneal inoculation of standard doses of half a loop) has been made by Piras (1913) He found that on the average the strains isolated from carriers possessed much less virulence than the cultures from patients. Whenever the growths initially isolated from carriers were virulent for the test animals, subsequently made cultures proved less virulent and, very often, finally quite avirulent. In Piras s opinion this diminution and eventual loss of virulence of the cholera strains isolated from healthy carriers accounted *partly for* the comparative harmlessness of the latter *It is of interest* to add that tests with cultures successively isolated from cholera convalescents also indicated a gradual loss of virulence, part of the strains finally becoming avirulent

Discussing the problem of a lessened virulence or avirulence of the cholera vibrios excreted by carriers Pollitzer (1952) pointed to laboratory

of transport, arrived in West Pakistan from all parts of India without being subjected to quarantine measures no importation of cholera took place

Claims were made by one member of the expert committee that cholera carriers particularly contact carriers, had played a role in the 1947 Egyptian outbreak Dealing with this contention the other experts stressed that

"The observations made in Ceylon, China, India, and Indochina during many years, on the other hand, do not point to contact carriers as playing a significant role. The trend of opinion in the committee was to the effect that contact carriers do not play a significant role in the spread of the infection."

It is important to add that as admitted by Kamal (1951) cholera convalescents even though they were usually discharged 12 14 days after onset of the disease, i e at a time when their stools were not necessarily free from *V cholerae* played no role in the spread of the 1947 Egyptian outbreak. Therefore as concluded by Pollitzer (1952) there seems no reason

"to revise the opinion held by most experts with experience in areas where cholera is endemic or frequent, that only acute carriers, that is, individuals late in the incubation stage, those actually ill and possibly also those in early convalescence, are instrumental in spreading the infection."

Sex and age incidence

The task of evaluating the statements made by numerous writers regarding the comparative frequency with which cholera occurs in the two sexes and in the various age groups is rather difficult in that (*a*) the size of the samples on which the various authors base their conclusions varies greatly thus rendering their statistical significance different and (*b*) worse still most observers merely quoted statistics regarding the sex and age incidence in the cholera outbreaks observed by them without correlating these figures with those showing the comparative frequency of members of the two sexes and of the different age groups in the general population

A closer study shows, however that these objections to the statistics available in regard to the sex and age incidence of cholera are of theoretical rather than practical importance because the most marked variance of the data recorded in these respects by the different observers speaks strongly in favour of the assumption that extrinsic factors rather than intrinsic causes are at work to lead in some epidemics to a markedly more frequent incidence of the disease in males or in children, while other outbreaks are characterized by diametrically opposite features While thus, in view of the markedly different extrinsic conditions under which the various cholera outbreaks evolve no general rules can be laid down it seems legitimate to state that (*a*) in a majority of the cholera outbreaks both sexes are affected in a comparable manner and (*b*) the opinion held by some workers that the infection is particularly rampant among young children has not been

of epidemic cholera are patients suffering from the disease, in the acute stage for about 4 days also some though to a much lesser extent in the convalescing stage for about 14 days and perhaps a few in the incubation period for a few days."

The large-scale inquiries under the auspices of the Office International d Hygiène Publique (see Couvy 1933 Stewart, 1933) led to similar conclusions, according to Taylor (1941) the evidence suggesting on the whole

"that with a very short persistence of *V cholerae* in the intestinal tract of the convalescent or contact carrier it was unlikely that the carrier was responsible for transmitting infection at any prolonged interval after the primary infection and consequently to places remote from cholera infected areas."

The results recorded above obtained before fully specific O sera had become available for the laboratory diagnosis of cholera, were confirmed by further observations made with the aid of such sera by Read & Pandit (1941) who as Taylor summarized, came to the conclusion that

"the detection of a carrier before the onset of a case in the vicinity was not accomplished and positive evidence was not obtained incriminating a carrier as the source of infection."

Seal (1945) discussing the problem of cholera endemicity in Bengal came to identical conclusions He noted that cholera vibrios could not be isolated from the stools of the general population or from water in the endemic areas except in direct relation to cholera patients, and considered contact carriers and water to be infective agents " for short periods and a short range " only

The question whether or not and to what extent carriers play a role in the spread of cholera was once more the subject of considerable debate at the 1951 session of the WHO Expert Committee on Cholera (World Health Organization 1952) and the meetings held in connexion with this.

Those experts with long experience in India or countries farther east maintained that cholera carriers were of no epidemiological importance *Attention was drawn in this connexion to the important observations* recorded by Nicholls (1953) under the title " Carriers of *V cholerae* who enter Ceylon from South India "

As summarized by Pollitzer (1952) Nicholls calculated that during the period 1924-33 at least 200 cholera carriers must have arrived in Ceylon during a year of average immigration It was known, on the other hand that during the same period there were only ten occurrences of cholera in the areas to which the majority of carriers went. Nine of these cholera manifestations were due to the arrival of *incubatory* carriers the origin of the tenth outbreak could not be elucidated Nicholls concluded, therefore, that the great majority of the carriers must have been excreting avirulent vibrios.

It was also emphasized that the observations made in West Pakistan since the partition of India fully supported the experiences in Ceylon Though hosts of immigrants or other travellers using all available means

infection among the staff of adequately managed cholera hospitals are rare exceptions nowadays if they occur at all Such an absence of infection among hospital staffs was frequently observed even long before the discovery of *V cholerae* but as summarized by Griesinger (1857) in other early cholera outbreaks there was a high incidence of such infections In Griesinger's opinion the reason for the difference was that the standards of cleanliness and sanitation adopted in the various hospitals varied considerably and particularly that

"rapid removal and disinfection of the evacuations are sometimes resorted to and sometimes omitted that the medical personnel is sometimes urged to watch over their health and to submit to rapid treatment of any diarrhoea and at other times neglects such precautions that old and overworked individuals leading an immoderate life sometimes function as nurses that, briefly in some instances various auxiliary factors either exert an untoward influence or grant protection." [Trans.]

Similarly Flügge (1893) pointed out that on the one hand untrained nurses were apt to fall a prey to the infection while on the other hand

"Physicians, the trained nursing staff of hospitals, persons educated to cleanliness, who do not touch their mouth or their food with unclean fingers and do not keep their food in the sickroom, are not exposed to the infection." [Trans.]

Factors Governing the Spread of Cholera over a Distance

General considerations

While many authorities are in agreement with the dictum of Greig (1929) that "the spread of cholera is effected by man himself" some observers have maintained that long-distance transport of the infection may be effected also by other means among which the following deserve mention

Infected water courses

It has been claimed or at least implied by a few writers that rivers or other water courses if they become contaminated with *V cholerae* may serve as a transport vehicle for the organisms This was upheld by Strong (1944) for instance who stated that

"In countries which lie adjacent to endemic centres of infection, the disease may spread considerable distances by an infected water supply Thus in India the infection has been carried by the River Cauvery for approximately 18 miles [30 km] to the Madras Presidency The infection was also said to be carried by Lake Five (the source of the water supply) which became infected through water pipes, for a distance of at least 11 miles [18 km]. Also in Mesopotamia cholera infection has apparently travelled long distances down the Tigris River"

As noted already Mathew (1949) dealing with the epidemiology of cholera in Madras Province (now Madras State) pointed to an ominous role

confirmed by many other observations Particularly noteworthy in this respect is the following statement by Kamal (1951)

"I have studied the morbidity amongst the age groups 0-1 and 1 5 during the 1947 epidemic. While infants and children comprise 13.24 / of the total population at risk, yet the number of cases that happened amongst them amounted only to 4.57 / and while the general morbidity rate per 100 000 was 103 the same rate for the age group 0-5 was 34 only and for those above five was 113

"These figures show clearly that although children stand the same risk of infection, yet the disease incidence amongst them is low"

Similar views were expressed by several other authors and it is usually held that cholera is as a rule most rampant among adults within the age limits of about 20 years to 40 or 50 years Commenting upon observations made in this respect in 1848-49 at London Snow (1855) stated that

"The greater part of the female population remain almost constantly at home, and take their meals at home, whilst a considerable number of the men move about in following their occupations, and take both food and drink at a variety of places consequently in the early part of an epidemic, when the disease only exists in a few spots, the male part of the population is most liable to come within the operation of the morbid poison but at a later period of the epidemic, when the cholera is more generally diffused, it may reach those who stay at home as readily as those who move about and in addition to the risk which the women share with the men, they have the additional one of being engaged in attending on the sick."

Racial incidence

As manifested by its history cholera has been able to cause outbreaks of identically wide spread and equally great severity among practically all races inhabiting both the old and the new world That no racial insusceptibility to this infection exists is likewise manifested by the severe toll formerly exacted by cholera among the British, especially among British troops, stationed in India. It is true that nowadays adequate standards of living and due attention to the precepts of hygiene are apt to go a long way in keeping foreigners resident in the Eastern cholera areas free from the infection. However a similar freedom from the disease may likewise be enjoyed by the strata of the local populations with standards of living and hygienic habits comparable to those in the Western world. Thus, as has been discussed already cholera has become mainly a disease affecting the underprivileged classes of the population, who alas, are not rarely even unable to pay for the pure water supplied by the waterworks and have thus to rely on unsuitable and easily contaminable other sources of water supply

Occupational incidence

As with the freedom from the disease at present enjoyed by the well situated and reasonably careful classes of the population so instances of

played in the irrigated part of this area by the smaller irrigation channels These he explained,

"pass through every village in the area. Almost each house has direct access to the channel. The channel water is grossly contaminated by personal ablution, washing of clothes and vessels, washing of animals, etc. Even the clothing and bedding soiled with cholera excreta are washed in them and yet the people drink this water as such as they find it more tasty than the well water It is a common experience that once any of these villages is infected with cholera, all villages situated lower down on the same canal or channel will be affected one after the other in quick succession. The rapid dissemination of cholera infection in canal-irrigated areas and its greater prevalence there are the natural consequence."

As stated by Benjamin (1949) a role similar to that of the Madras irrigation channels was played in the spread of cholera by the large number of rivers and their tributaries intersecting the districts on the Deccan Plateau of Bombay State There also it was

"observed repeatedly that once a town or village on the bank of a river or stream is infected, other villages downstream of the river usually get infected rapidly the distance to which infection spreads varies (*a*) with the size of the village or town first infected and (*b*) the duration of the infection in that village."

As Benjamin added, widespread outbreaks of "riverine" cholera produced in this manner were characterized by a distribution of the infection

"in the form of a band with the river in the centre of the band and an almost equal area consisting of villages on either side of the river but at a distance from it the latter being infected by infection due to communication with the infected riverside villages. In non-riverine areas, the infection spreads radially round the initial infection, the radius varying with the size of the original town or village infected, its importance as a trade centre and the facilities for communication."

Interesting as these observations are the opinion that contamination of water courses with *V cholerae* plays an important *direct* role in the long-distance spread of the infection has not been universally accepted Robertson & Pollitzer (1939) in particular who had good opportunities for similar observations in China, maintained in this connexion

"We have no evidence suggesting that the causative organism has been carried over any appreciable distance by the waterways themselves. In fact, infection, though often travelling along them, has frequently spread upstream rather than downstream. Certainly however water borne traffic was one of the principal means of broadcasting cholera. On the other hand one must not overlook the fact that motor roads and other routes of land traffic generally run parallel with the waterways the principal settlements lying usually on both. It was thus sometimes difficult to decide by what particular route infection had reached a given settlement."

Notwithstanding these objections one should not categorically rule out the possibility that contamination of water courses with *V cholerae* may be responsible for a spread of the infection to riverine settlements lying a short distance downstream from the originally affected localities.

(corresponding to a trip of 300–400 km) admitted that the infection could be carried over wider distances by caravans passing through well populated areas

(2) Railways

The great importance of railways for the spread of cholera was well illustrated by observations recorded at the 1885 cholera conference by Koch according to which

"The Punjab belonged to those parts of India which formerly suffered least from cholera. From the year 1820 in which the first reliably authenticated cholera epidemic came into the Punjab, up to the sixties i.e., in a period of about 40 years the province had only 5 epidemics 1820 1827 1845 1852 and 1855 Then railway traffic was opened. From then onwards a comparatively large number of epidemics followed regularly 1861 1862, 1865 1867 1869 1872, 1875 1879 1881 Thus suddenly from 1861 onwards the behaviour of cholera in the Punjab became changed. The population remained the same, and the meteorological conditions did not change only the traffic with the cholera focus in Bengal was accelerated." [Trans.]

Agreement with the opinion of Koch that railway traffic played a most important role in the long-distance spread of cholera was expressed by several other early observers some of whom pointed in this respect particularly to the causation of a quite considerable epidemic at Altenburg in Saxony in 1865 through the arrival of a woman with a cholera affected child from Odessa in Russia

It is reassuring to note that some of the modern observers are inclined to lay less stress on the role played by railway traffic in the spread of cholera. Thus Napier (1946), dealing with the conditions met with in India maintained

" that normal railway travel on business or pleasure does not tend to spread the disease to any great extent on account of the control that can be exercised over passengers and that, though the sanitary arrangements are far from perfect, especially at the small stations, there are latrines and a safe water supply Such travellers of course come from all grades of society but even the poorest are seldom destitute and the fact that they are travelling usually indicates that they can afford the ordinary necessities of life."

(3) Ships

While it was always agreed that the local traffic by small craft played an important role in the propagation of cholera, the question to what extent ships undertaking long voyages were of importance for the spread of the infection was in the past the subject of much debate The anticontagionists tried to prove that cholera manifestations on sea going ships were rare and therefore of no epidemiological significance However as pointed out by Koch (1885) it was wrong in this respect to take into account all cholera-infected ships, including those leaving ports which were only occasionally affected by the disease instead of focussing attention upon

of contaminated flies, carried, for instance, by railway trains Certainly however such a transfer of the infection if it takes place at all, must be rather rare as compared with the usual mode of cholera propagation through persons who leave affected localities when already ill or when incubating the disease to fall a prey to it either in transit or after they have arrived at their destination. The factors responsible for the speed and the intensity with which such a long-distance spread of cholera through human agency takes place will now be given attention.

Spread by infected individuals using various methods of transport

In the course of an interesting discussion of cholera epidemiology Hart and his colleagues (1910) pointed out that man

"can but carry the disease so far as he is able to travel between receiving the infection and being laid low What we find then on comparing the march of the earlier epidemics of cholera with those that have occurred in more recent years, is that whereas when travel was slow the disease swept steadily forward, occupying the land as it advanced in later times it has bounded forward with long strides, occupying outposts far ahead of infected areas by means of railway and steamboat communication, and then from these outlying foci of infection, has spread in both directions, coalescing perhaps at a much later date with the main body of the epidemic which has slowly advanced across country from the earlier centres."

While this statement is of great value in so far as it does justice to the ability of cholera to make long-distance sprints if modern means of communication are available it might give the wrong impression that the slow contiguous spread of the infection is a quite uniform process. Actually as will be discussed below in this mode of spread as well initial foci may be created in places particularly suitable for inroads of the infection and from these centres the disease may be carried to the surrounding localities One might thus say that a spread of the infection by shorter or longer relay stages is typical of the propagation of cholera.

Though, as pointed out by some authors, first perhaps by Greig (1919) a rapid spread of cholera might be effected by air traffic, as far as the present writer is aware this fear has not been substantiated thus far It is permissible, therefore to focus attention upon other means of communication.

(1) Caravans

Dealing with the problem of the spread of cholera by caravans, Duguet (1931) stated that

"The sterilizing effect of caravans has always been stressed. Indeed, upon seven occasions, the caravan of Syria, starting from Medina, left with cholera and the malady became extinct en route without ever being imported into Damascus." [Trans.]

However in a subsequent publication, Duguet (1932) while noting that as a rule cholera persisted in the Medina caravans only for 10-15 days

which is either the birthplace of a saint or where his ashes lie buried. They sometimes travel a distance of more than three or four hundred miles."

As pointed out by Rao when dealing with the special case of the *palki* proceeding to Pandharpur (in Bombay State) such processions formed not only a safe but also a cheap means of making a pilgrimage especially since the participants used to be fed en route by the devotees of the saint in whose honour the pilgrimage was made. Therefore they continued to be popular even though much more rapid means of transport had become available.

Among the numerous important pilgrim festivals and fairs held—mostly every year—in various parts of India (see list by Lal 1937) the following deserve special mention.

(*a*) As summarized by Banerjea (1951) about 400 fairs attracting a total of over 12 million people, were held annually in the *United Provinces* (now *Uttar Pradesh*). Of these, Banerjea continued,

"116 fairs with a total gathering of 287 000 are held mostly in eastern districts in the months of March and April (when meteorological conditions become favourable for the spread of the disease). Besides these, Kumbh and Ardh-Kumbh fairs at Hardwar and Allahabad, the two largest fairs in India, alternate every sixth year at each place. A large pilgrimage, therefore, occurs at one or the other every fourth year. Kumbh and Ardh-Kumbh fairs attract a gathering of nearly three and two million respectively at Allahabad and about one million and half million respectively at Hardwar."

The dismal influence which these large gatherings exerted on the incidence of cholera in the United Provinces is well illustrated by the following data furnished by Banerjea, showing the effect of Kumbh (K) and Ardh Kumbh (A) fairs on cholera mortality.

Hardwar			*Allahabad*		
Year	*Category*	*Number of deaths*	*Year*	*Category*	*Number of deaths*
1879	K	35 892	1882	K	89 372
1885	A	63 457	1888	A	18 704
1891	K	169 013	1894	K	178 079
1897	A	44 208	1900	A	84 960
1903	K	47 159	1906	K	149 549
1909	A	21 823	1912	A	18 894
1915	K	90 508	1918	K	119 746
1921	A	149 667	1924	A	67 000
1927	K	28 285	1930	K	61 334
1933	A	1 915	1936	A	6 793
1938	K	70 622	1942	K	7 662
1945	A	77 345	1948	A	52 604

This epidemic was said not to have been connected with the Ardh-Kumbh.

As Banerjea added, the average death-rate from cholera in the years during which no Kumbhs or Ardh Kumbhs were held was 1.6 times lower than that during the festival years.

marized in the chapter just mentioned outbreaks continued to occur among the pilgrims at Mecca at frequent intervals—according to Duguet (1931) thus

Period	*Number of epidemics*
1855-1866	9
1881 1883	3
1890-1893	4
1907 1912	4

Due invariably to the early arrival of some pilgrims from the Eastern cholera foci (mostly by the sea route) the Mecca epidemics gained impetus when large numbers of pilgrims had crowded together during the festivals, reaching their maximum, Duguet maintained, either in spring and early summer (May-July) or in autumn and winter (October January) They continued to be a serious menace for the countries to the west until the progress of the disease was effectively barred through the establishment of a quarantine camp at El Tor

(2) Pilgrimages and religious festivals in India As aptly stated by Banerjea (1951)

"The association of cholera with pilgrimages, fairs and festivals is well-known. These have acquired notoriety and are considered by health authorities as starting points of wide-spread epidemics. If one of these fairs of some respectable size happens to take place in the cholera season, then, but for the most stringent sanitary precautions, a severe outbreak of cholera is certain and is spread widely by the dispersing crowds. Smaller fairs and festivals can also be dangerous because of the less satisfactory arrangements and control. As pointed out by Lal (1937), not only is the place of congregation the danger spot, but the nodal points along the pilgrim routes are sources of equal anxiety to the health administration"

According to the statements of authors like Lal (1937) and Banerjea (1951) the festival centres in India fall into two classes—namely (1) places of perennial pilgrimage, and (2) temporary camps specially erected for periodical fairs and festivals

As explained by Lal,

"The former possess some special religious sanctity apart from the occurrence of holy days. They continually attract pious people from different places. Kalighat, Benares, Puri, Rameswaram and many others may be cited as examples of such centres. The latter type of centre comes into prominence only on certain days in the year and, more particularly periodically after a number of years. The attractions of these places are as much secular as religious. Kumbhs and Adha Kumbhs at Hardwar and Allahabad are the best examples."

Pilgrimages of a peculiar kind possessing an epidemiological importance of their own and therefore requiring special methods for their control were the so-called "moving religious festivals" or *palkies* a term derived according to Rao (1947) from the word "palanquin" because these

"were in this particular case used or intended for carrying the wooden sandals of some well-known saint to a central place of pilgrimage These *palkies* start from a place

several localities. This was the case with the cholera invasion of 1947 Not a single case of cholera was recorded at the Adi Ammavasi festival at Rameswaram which took place in July 1947 and yet several districts in the south were infected by returning pilgrims and a severe epidemic was started The source of the infection was traced to a party of pilgrims from outside the province."

(*e*) Dealing with the cholera situation in *Bombay State* Benjamin (1949) recorded that among the numerous places of pilgrimage in this area that at Pandharpur was the largest Fairs were held there almost every quarter but the two most important ones which were visited by a large concourse of people from almost all the Marathi speaking regions of Bombay State as well as the Central Provinces and Hyderabad State were the Ashadi and the Kartiki fairs the former being held in June or July the latter in November As has been noted before a peculiar feature of the Ashadi fair was that it was the goal of some organized processions (*palkies*) starting from localities outside as well as inside Bombay State and attended at the time they reached Pandharpur, by up to twenty thousand people. It is obvious that these processions which repeatedly had to camp en route were in the past a dangerous means of spreading cholera. However as will be discussed in the following chapter, most gratifying progress has been made within recent years in coping with these situations and controlling the pilgrimages in general

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As proved by statistics of Rogers (1928) and by other observations, the prevalence of cholera in the United Provinces during the Kumbh and Ardh Kumbh years inevitably led to an exacerbation of the cholera incidence in India in general Particularly important was that, owing to its geographical position in the north-west of the United Provinces, a high incidence of the disease in Hardwar almost invariably led to a serious cholera situation in the Punjab and that the prevalence of the infection in the latter area was apt to be responsible for a further westward progress of the scourge resulting in its pandemic spread Thus as noted in Chapter 1 the Hardwar pilgrimage of 1826 and the subsequent invasion of the Punjab were responsible for the second cholera pandemic gaining impetus in 1829 Banerjea (1951) asserted on the authority of Wu Lien-teh (1934) that the pandemics of 1866-70 and of 1892 95 likewise

"spread from Hardwar pilgrims to the northwesterly province of Punjab in India and by the overland route spread to Afghanistan, Persia, Southern Russia and finally invaded both Europe and America."

(*b*) Duggal (1949) discussing the cholera situation in *Bihar* stated that, besides religious festivals and cattle fairs of solely local importance some festivals of interprovincial importance were also held in Bihar the pilgrim centres at Deoghar and Gaya, for instance attracting visitors from all parts of India. Unfortunately most of the religious festivals in Bihar fell in the cholera season, thus being likely to serve as starting points or distributing centres of widespread epidemics.

(*c*) Dealing with the cholera situation in *Orissa* situated south of Bihar Hajra (1949) declared that

"The large number of perennial festival centres, for which the province is most popular forms a strong foothold for cholera. As an instance I may cite the case of the car festival at Puri. This festival attracts the largest number of pilgrims varying from 1½ to two lakhs [i.e., 150 000-200 000] not only from the province but also from the whole of India The festival occurs during June and July just as the south-west monsoon sets in and fly breeding is at its maximum. All these factors contribute to the introduction and spread of cholera infection in the province This might as well have been responsible for some of the epidemics in other provinces in certain years."

(*d*) As described by Mathew (1949) *Madras State* had over a thousand festival centres. Though many of them were merely of local importance, nevertheless they could play a dangerous role if they became infected with cholera, because, as Mathew maintained with much reason,

"Although it is possible to make elaborate sanitary arrangements at the festival centre itself, the innumerable routes by which the pilgrims travel to the festival centre and back are a difficult problem especially in regard to water supply and conservancy"

Thus, Mathew found,

"It is a common experience to find that although the festival itself has passed off without any cholera outbreak, the returning pilgrims have conveyed the infection to

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Chapter 11

PREVENTION AND CONTROL

PREVENTION

General agreement has been reached that, as Sticker (1912) aptly put it,

"The cholera free times are the times of anti-cholera work. The true amelioration of all conditions of humankind is effected in peace times through arrangements and adoption of habits inimical to the invasion and entrenchment of noxious factors in general and of epidemics in particular. The best, indeed the only justified, sanitary police is that which countenances the approach of diseases with a calm conscience because it feels entitled to say that all humanly possible precautions have been taken." [Trans.]

The measures called for to prevent the invasion and spread of cholera may be specified as follows:

Provision of Permanently Safe Water Supplies

The question whether, as is now generally accepted, the supply of permanently safe drinking-water plays a paramount role in cholera prophylaxis, has been much debated in the past. However, as Liebermeister stated when writing in 1896:

"Pettenkofer's dogma, according to which no infection is produced through drinking-water, has been so decisively invalidated through the sad experiences of the last years, that no dialectics will once more give credence to it. Provision of safe drinking-water not exposed to contamination, forms, therefore, one of the prime tasks of cholera prevention." [Trans.]

Liebermeister added with great reason that the question how to procure such safe water supplies, depending as it did upon varying local conditions, could not be answered in a uniformly valid manner. In accordance with this view, which has been generally accepted, it is necessary to pay separate attention to the procurement of safe water supplies from (1) waterworks and (2) properly constructed and adequately used wells.

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Authority	*Findings*		
Mendelson & Tait (1921) *(continued)*	river where safe water supplies were lacking. The morbidity and mortality figures per 1000 of the population were as follows		
		Cholera morbidity	*Cholera mortality*
	East side	1 376	0 800
	West side	9 42	4.958
Hoops (1935)	As stated by Hoops at the 1934 conference of the Far Eastern Association of Tropical Medicine, "cholera used to be endemic in the Malay Peninsula but had disappeared in every State on the introduction of a water supply to the principal centres of population" Hoops stressed in this connexion that the pure water was issued free from standpipes to the poor		

The validity of the findings quoted above has been fully confirmed by recent observations Thus there can be hardly any doubt that the availability of pure waterworks water was the most important reason why as Shousha (1948) put it in his account on the 1947 cholera epidemic in Egypt "the disease has failed to establish itself in any of the towns provided with satisfactory sanitary accommodation" Similarly Benjamin (1949) dealing with the problem of cholera control in Bombay State, recorded that

"The provision of a pipe water supply to towns has resulted in most cases in a marked improvement in the incidence of cholera. In a few cases where the water supply is insufficient and where the people take water from a river near the town, the improvement is slight."

In accordance with these observations Subrahmanyan (1951) came to the conclusion based on statistical computations, that

"Whether the district was highly endemic or not, the town with a piped water supply was better protected against cholera than the rural district under the same conditions, both in East Bengal and West Bengal."

While thus there can be no doubt that the installation of waterworks in communities threatened or frequently affected by cholera is a boon of paramount value constant vigilance must be exerted to ensure that the supposedly pure water supplies thus made available are really of the indispensably high standard. Avoiding false economies emphasis must be laid therefore upon proper construction and equipment of the waterworks Particularly if the supplies to be purified are drawn from rivers or other surface waters it is most desirable to use chemical treatment, especially chlorination of the water in addition to adequate filtration. If chlorination is done serious attention ought to be given to the advisability of increasing the chlorine content of the water to a higher value—up to two parts per million—at times when cholera threatens or is present This useful precaution was adopted for instance, in some of the Chinese cities, particularly in Shanghai without meeting with serious opposition on the part of the consumers.

Waterworks

When dealing with the problem of water borne infection in the preceding chapter attention was drawn to several observations showing that the pollution of waterworks water with *V cholerae* either due to the use of unfiltered supplies or resulting from faulty functioning of the filter plants was the cause of explosive cholera outbreaks However these occasional disasters cannot invalidate far more ample evidence to the effect that as a rule the opening of properly functioning waterworks led to a marked decline, sometimes even to the disappearance of cholera in the communities concerned Emphasis upon this most important method of cholera prevention was laid by Macnamara as early as 1876 when he stated that he could not

"but believe that the introduction of a pure water supply into Calcutta has been the immediate cause affecting the diminution in the death rate from cholera which has existed during the past 5 years amongst the inhabitants of the place." [1]

Further noteworthy observations made to the same effect may thus be summarized

Authority	*Findings*
Flügge (1893)	From 1831 to 1867 Breslau (then in Prussia) had eleven cholera outbreaks, most severe in part. However in 1873 i.e., two years after the opening of the waterworks, in spite of repeated importations of the infection, the number of cholera cases totalled no more than 59
Blumenthal (1909) Sticker (1912)	The cholera incidence in Moscow decreased incessantly *pari passu* with enlargement and improvement of the waterworks originally opened in 1805 In 1908, after ample and fully reliable waterworks water supplies had become available, only 16 persons died of cholera as against over 7000 in St. Petersburg, the water supply system of which continued to be quite unsatisfactory
Harris (1913)	The provision of piped water supplies in various towns of the United Provinces (now Uttar Pradesh) of India led to a great reduction of the cholera mortality That the infection did not disappear altogether was due to extrinsic causes, such as the continued use of other sources of water supply and the presence of numerous pilgrims.
Mendelson & Tait (1921)	Observing a 1919-20 cholera outbreak at Bangkok, Thailand, Mendelson & Taid noted a most marked reduction of the cholera incidence in the eastern part of the town, which was provided with a good waterworks system, whereas the infection was much more rampant on the west side across the

[1] As noted in the preceding chapter Koch, speaking at the 1884 Cholera Conference in Berlin, also referred to the beneficial effect exerted by the opening of the Calcutta waterworks in 1870

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Mendelson & Tait (1921) *(continued)*	river where safe water supplies were lacking. The morbidity and mortality figures per 1000 of the population were as follows		
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Either the waterworks must be provided with adequate laboratory facilities of their own or samples of their raw and their processed water must be sent frequently preferably daily to public health laboratories in or near the communities in question Great care must be taken to draw samples of the purified water not only from the main in the works, but also from peripheral taps, because—contrary to not rarely held beliefs—tampering with peripheral pipes as may occur for instance when electrical or telephone cables are laid or other digging operations are undertaken in the streets, may lead to localized peripheral pollution of adequately processed waterworks water Observations on this point seem to have been made by Mendelson & Tait (1921) for instance, who stated that

"the efficiency of taps was not always up to standard, but this was always found to be a local affair with the tap examined, and in no way the result of the filters."

The present writer even knows of an instance in which wilful tampering with a pipeline leading to a refugee camp so as to obtain water for an adjacent newly established small camp of "squatter" refugees led to an incipient cholera outbreak among the latter obviously due to contamination of the superficially laid pipes in the cholera affected slum area in question. Repair of the permanent pipe system and installation of a proper water supply for the squatters promptly led to the disappearance of the infection among the latter

Reference has already been made to the observations of Koch (1884) and some subsequent workers who had shown that the opening of water works, though invariably leading to a marked decrease in the incidence of cholera, did not as a rule lead to complete disappearance of the infection from the communities concerned, because the safe water supplies had not been made available on a really community wide scale or because, if water works water was laid on everywhere, people with restricted means were often unable to pay for it and were thus forced to rely upon unsafe sources of water Attention has been drawn on the other hand, to statements of Hoops (1935) according to whom the universal use in the principal cities of Malaya of waterworks water which was issued free from standpipes to the poor had led to complete disappearance of cholera. These observations speak for themselves and clearly demonstrate the imperative need to supply safe water to rich and poor alike without any regard for narrow fiscal policies It is greatly preferable for permanent arrangements to be made in this respect, but, as will be discussed later failing a permanent provision of safe water for all inhabitants, temporary arrangements for its supply can be made at times when cholera is present or imminent.

While in view of the obvious importance of safe water not only for the purposes of cholera prevention but also for attaining a satisfactory state of public health in general an ever increasing number of cities and towns in most parts of the world is provided with adequate waterworks, unfortunately

the cost of procuring such safe water supplies for villages or for groups of settlements in rural areas as well is as a rule prohibitive. However, while funds for the erection of waterworks in rural localities may be unobtainable there ought to be no real difficulty in raising sums sufficient for the systematic improvement of the well water supplies, which will now receive due attention.

Wells

Though wells—which serve as one of the most important sources of water supply in cholera affected rural areas—may be classified in various ways, it seems best for the purposes of the present disquisition to pay separate attention to (*a*) dug, invariably shallow, wells and (*b*) tube wells which, as will be discussed below, may be drilled to depths varying from those usual in the case of dug wells (as a rule not more than 30 feet or about 10 metres) to hundreds of feet.

Before paying further attention to these types of wells it is important to state that, while in the past great emphasis was laid upon the danger of subsoil pollution of their water, according to subsequent investigations such contamination takes place to a far lesser extent than was formerly feared. Ably discussing this point, Subrahmanyan and colleagues (1948) stated in their profound studies on rural water supplies that the danger of well contamination through the soil

"has been emphasized and perhaps over-emphasized by sanitarians in all countries. Caldwell & Parr (1937) and Dyer [and co-workers (1945)] have found that in sandy or clayey soils free from fissures, bacteria from such an obvious source of pollution as the borehole latrine do not travel more than 10 ft. to 25 ft. [3 m–7.5 m] in the direction of the flow when the rate of withdrawal of groundwater is not high, and therefore the chances of contamination of wells subject to such usage as may be expected in rural areas are not so great as has been believed in the past."

However, the danger of surface pollution of unprotected or insufficiently protected wells is great indeed and, as has already been mentioned in the preceding chapter, the often used method of collecting the water of shallow wells with the aid of buckets or other vessels is apt to render even properly installed wells highly dangerous. Means of counteracting such possible pollution and surface contamination in general will be discussed in the following disquisition on the types of well commonly used in the cholera affected areas.

Shallow dug wells

The character of the primitive shallow wells still met with in the cholera areas is exemplified by the following description by Bishop (1913):

"The 'pot well' common to this part of Bengal is an earthen shaft lined by burned pot rings with a diameter of 2 feet 9 inches [80 cm]. Such wells are sunk usually in the dry season, and are carried down to a depth of between 20 and 30 feet [6 m–9 m], *i.e.* until a depth of water of 4 to 6 feet [1.2 m–1.8 m] is obtained. The well lining is generally built up some 3 feet [about 1 m] above the ground surface and supported by a small earth ramp."

Fortunately owing to the particularly great interest now paid in the cholera affected areas to improvements of the rural water supplies, this primitive type of shallow dug well tends to be replaced by better constructed wells. Thus Subrahmanyan and co-workers (1948) found it possible to classify the open wells they met in the course of their studies in another part of Bengal in the following five sanitary categories

(*a*) Parapet, apron and drain satisfactory

(*b*) Parapet and apron satisfactory but drain for waste water less than 5 feet [1.5 m] long or broken or not provided

(*c*) Parapet and drain satisfactory but apron unsatisfactory

(*d*) Only parapet or apron or drain satisfactory

(*e*) Parapet, apron and drain all unsatisfactory

The reason why the character of the wells varied so considerably and why evidently a good number of them were in an unsatisfactory condition was perhaps that most of the shallow open wells seen by Subrahmanyan and colleagues were privately owned. As they added, these wells were also less popular than tube wells for drinking-water supply Almost none of the shallow wells was fitted with covers or provided with pumps so that water was drawn from some of them with the aid of a pulley and bucket, while in others it was obtained with buckets let down by hand.

An interesting attempt to improve the system of water delivery from shallow wells with the aid of locally available means has been described and illustrated by Khan (1934) He proposed the use of a collecting board made in the shape of a boat by joining several wooden boards of convenient size this board projected on one side into the lumen of the well-shaft and, slightly sloping, was connected on the outside of the well parapet with a delivery tap situated about 1½ feet (50 cm) above ground level. The functioning of this well which was provided with a detachable cover made of boards and from which water was drawn in an iron bucket with the aid of a rope and pulley was thus described by Khan

"As the bucket is pulled up and has reached above the level of the collecting board, the handle of the bucket engages in the loop of the push bar [1] At this moment the push bar is pulled by the handle for a convenient distance, and at the same time a slight movement of the drawing pulley in the reverse direction, as in lowering the bucket, allows the bucket to be drawn on to the upper surface of the collecting board. The bucket is released there by a further movement of the drawing pulley As the bottom of the bucket is pointed, it falls on its side, and if the collecting board is so made as to allow this to occur without impediment, the bucket is almost completely emptied, and the water runs out of the delivery tap After the bucket is emptied, it is lifted up by a slight turn of the drawing pulley in the right direction (*viz* in the direction of drawing up the bucket) and pushed out by the push bar clear of the collecting board. It may then be lowered into the well again."

This is a bar (made of wood or iron) with a loop at one end through which the rope passes It was used in the above-described manner to pull the bucket on to the collecting board and to push it off again after the water had been discharged.

There can be no doubt that the scheme for well improvement recommended by Khan was theoretically sound. At the same time, however, one cannot help noting that the apparatus recommended by him was somewhat difficult to handle and one must also fear that it was by no means easy to keep it in good working order. It was probably for these reasons that this ingenious scheme for well improvement did not attract attention.

Subrahmanyan and co-workers (1948) examining 44 shallow open wells in the Singur and Balarambati districts of West Bengal found the bacterial content of these water supply sources, as measured by the number of gas-forming bacilli in 100-ml samples, distressingly high but noted a considerable decrease in this bacterial contamination during the cold season. In order to attempt improvement of the wells, these workers provided seven of the shallow open wells with sheet iron covers as well as with deep-well pattern pumps, while seven others of these wells also fitted with such pumps were left uncovered. After a few months some more of the wells were protected by concrete covers as well as provided with pumps. The value of these various improvements was assessed through weekly determinations of the coli titre of the water of the wells, samples taken simultaneously from the unimproved wells serving as controls. Commenting upon the results of these comparative tests, Subrahmanyan and his colleagues stated the following:

> "Open shallow wells yield water that is definitely inferior to tube wells in bacteriological quality. The bulk of the pollution appears to be introduced directly from the surface through the open top and through buckets and ropes. It appears that very little of the contamination is introduced underground. The soil and the pottery rings and other lining of the open wells are, however, all conducive to the retention of any contamination that may be introduced. When a pump is fitted, there is a considerable improvement in the bacteriological quality of the water. There is a further improvement when the top is closed by a concrete cover and it is practicable to attain a standard of not more than 100 *coliforms* per 100 c.c. in 75 per cent of the samples. This is, however, far inferior to the practicable standard for tube wells and it is necessary to carry out further investigations on open wells."

There is no doubt that, making lavish use of concrete and other first class materials, it would be possible to construct shallow wells which, if they tapped a satisfactory source of water and were provided with suitable pumps, would prove fully adequate. Since, however, as will be discussed below, often quite satisfactory water supplies may be obtained with the aid of far more easily constructed and considerably cheaper shallow tube wells, one must wonder whether it would be justified to make provision for elaborate patterns of dug shallow wells under the conditions usually prevailing in the areas affected or threatened by cholera.

Tube wells

Bishop (1913), who was able to collect ample experience on the construction of tube wells when engaged in a cholera prevention scheme in

Bengal found that the usually recommended procedure of forcibly driving in the pipes used for the shallow type of this kind of well (25 feet or 7.5 m) with the aid of wrenches was unsatisfactory because as a result of this procedure the filter point of the pipes was apt to become damaged or tightly packed with earth Hence, he stated,

"The plan which we have found most successful has been to first sink a four inch [10 cm] outer pipe and, if water of good quality is found within 25 feet [7.5 m] of the surface, to introduce within this four inch bore hole a filter point with a sufficient length of two inch [5 cm] piping attached. The pump is then temporarily fitted and worked for some hours. If the resulting water supply is satisfactory the pump is detached and the outer four inch tubing withdrawn, the inner pipe being left permanently in position. The upper end of this is secured firmly by clamping between a couple of sleepers to which the pump when fixed is screwed."

If it was impossible to obtain a satisfactory water supply with this shallow type of well, the 4-inch pipe had to be driven down until a sandy layer bearing a good water supply was reached. Then an inner tube of 1¼ inch (3-cm) calibre was inserted and its upper end was connected with a suitable pump

An exhaustive investigation of 134 tube wells varying in depth from 50 to 250 feet (15 m-75 m) was made in the above-mentioned two districts of West Bengal by Subrahmanyan and colleagues As they stated, the *most important observation arising out of these studies was*

"that tube wells of moderate depth can serve as satisfactory sources for safe water supply in rural areas with soil conditions similar to those at Singur i.e. over a large part of the Gangetic plain and delta. They yield water that is little affected by comparatively insanitary conditions on the surface, change of seasons, heavy usage or even the use of priming water The water from the surface is not able to mix directly with the ground-water and the soil gets naturally compacted around the tube well so as to cut off surface contamination. There is little chance of the ground-water getting contaminated under the prevailing conditions of soil and usage."

It was thus practicable to attain and to maintain a bacteriological standard of not more than 10 coliforms per 100 ml in 80% of the samples taken from these wells As the authors added

"This standard appears to be consistent with the absence of gastro-intestinal diseases traceable to tube-well water and may be considered as the standard for adoption in judging the purity of rural water-supplies from tube-wells. If in any place the soil is likely to develop cracks and fissures, the surface water will be able to mix with the ground-water and the samples will naturally fail to come up to this standard."

While it was essential to chlorinate the pumps and wells whenever repairs had been made on the former it was found that "periodic chlorination of tube wells does not effect any lasting change in the quality of the water and appears to be unnecessary" Nevertheless the maintenance of the tube wells was difficult and expensive in that the pitcher type pumps commonly used for tube wells in India were rather liable to get out of order so that in the areas surveyed by Subrahmanyan and co-workers

the pumps of public tube wells needed an average of two repairs a year It was true that

"the time taken for actual repairs does not exceed 1 hour in 95 per cent of the cases and the cost of materials does not exceed Rs. 4-8-0 in 83 per cent of the cases. However taking the difficulties of road transportation into account one man is required to maintain 150 tube-wells and the total cost of maintenance is about Rs. 8 per tube well per annum." [1]

It might seem well to reduce this current expenditure by the installation of pumps of better construction but as the people are not rarely rather inconsiderate in handling the pumps, one cannot feel convinced that the initially greater outlay for a better type would be compensated for by decreased maintenance costs. No doubt however, better constructed pumps, even though not fully proof against damage, would break down less frequently thus reducing the times during which the people, being unable to get tube well water have to rely upon unprotected water supplies Therefore the problem of the pattern of pumps to be used in the areas affected or threatened by cholera deserves thorough study

In spite of the difficulties still existing there can be no doubt that the large-scale installation of adequate tube wells would be of cardinal importance for the prevention of cholera in rural areas in which it is not possible to provide for piped water supplies. Particular attention ought to be paid to the problem of making tube wells widely available in the cholera endemic areas, because, as Napier (1946) maintained with full reason "if all the inhabitants of these areas could be provided with a protected water supply it seems very probable that cholera could be stamped out"

Unfortunately in a part of the cholera-endemic areas especially on the islands in the Brahmaputra delta, an extremely high ground water level forms an unsurmountable impediment to the construction of wells Short of supplying piped water which at present at least seems out of the question, the installation of some kind of local filter plants seems the only possibility for the permanent provision of protected water supplies to the inhabitants of such localities This also is a problem which urgently calls for study

Other Improvements in Environmental Sanitation

It is historically interesting to note that the question whether the provision of protected water supplies or other improvements in environmental sanitation particularly a proper system of sewage disposal, were of paramount importance for the prevention of cholera has been the subject of considerable dissension in the past Pettenkofer and his adherents

[1] Rs 8 = US $1.80

(who as noted before did not believe that water supplies played an important role in the spread of the infection) stressed that in order to diminish the "local disposition" to the epidemic spread of cholera, an adequate system of sewage and refuse disposal must be given prime attention. However this view was not shared by Koch (1884) who pointed out that in Calcutta, where work on a proper sewage system had been commenced in 1865 from that year

"up to 1870 the effect of the continuously expanding drainage system on cholera mortality was not noticeable. But immediately the waterworks were opened [in 1870], cholera decreased and has continued since then on the average at one third of the previous level. However the incidence of cholera, which fell immediately a supply of wholesome drinking-water was introduced, has not been further lowered by the considerable extension of the drainage system that has occurred since 1870 Hence this favourable result has to be ascribed solely to the piped water system." [Trans.]

Koch's contention was supported by the observations of Mendelson & Tait (1921) As noted above these two workers found a low incidence of cholera in the parts of Bangkok which were provided with a pure water supply At the same time however they stated that in these precincts as well as in the city in general the night soil disposal was quite unsatisfactory a badly functioning bucket system being used.

It is further noteworthy that, as described by Subrahmanyan (1951) the small towns of Bengal, though provided with service privies and conservancy staffs, were nevertheless in a quite unsatisfactory state of environmental sanitation being "usually unclean, with flies, filth and sullage stagnating in the street drains" and thus dirtier as well as more overcrowded than the villages Still, as has been noted above, the cholera death rate was lower in the towns provided with piped water supplies than in the corresponding rural districts

While these observations leave no room for doubt that in order to prevent inroads of cholera emphasis has to be laid upon the provision of pure water supplies, it would be most unwise to neglect the erection of a second line of defence through making suitable arrangements for other sanitary improvements particularly a proper sewage system and adequate methods of refuse disposal. These measures in their turn will exert an important influence on the number of flies and thus reduce or even eliminate another potentially dangerous factor in the spread of cholera.

As will be discussed in the second part of this chapter the state of environmental sanitation in cholera affected localities is unfortunately even nowadays often so unsatisfactory that energetic temporary measures have to be adopted for its improvement However the possibility of making sometimes quite successful use of such emergency methods should never serve as an excuse for neglecting the adoption of long range programmes for the introduction of permanent sanitary improvements The fact that much interest is being taken nowadays in such permanent sanitary improve-

ments for reasons quite unconnected with the threat or the presence of the disease is of great benefit to cholera workers [1]

The choice of the permanent sanitary improvements to be adopted for purposes of cholera control depends upon the local conditions In rural areas in particular it is often out of the question to introduce a proper sewage system so that arrangements have to be made for family or for communal privies or, what is often more desirable for borehole latrines Apparently favouring the latter Subrahmanyan (1951) in a discussion of the anti-cholera programme adopted in the Singur health-demonstration area of West Bengal stated that

> "A drive was made to arouse the interest of the people in safe methods of excreta disposal About 2474 borehole latrines were put down in 7 years against 12 000 needed actually There was great apathy in the beginning. A good many latrines were not taken to the proper depth. Most of the borehole latrines were filled in about 1½ to 2½ years. Perhaps only about 600 may be functioning now but the demand for reboring and servicing of latrines has been increasing. The reboring is being done now New types of latrines are being developed, to overcome some of the defects in borehole latrines. The latrines are all for family not public use."

As Subrahmanyan added the cost of the excreta disposal programme was borne almost entirely by voluntary contributions of the people Concrete seats fitting the latrines were made available to them at cost price. Evidently, however he was in favour of the policy that materials as well as technical assistance for making the latrines should be supplied by the State and the necessary labour by the communities. While ready to admit that properly made latrines should eliminate the accessibility of the excreta to flies and the possibility of water pollution Subrahmanyan insisted that the latrines would be

> "of use in reducing cholera only if people learn to perform their ablution after defaecation in the latrine itself and give up the habit of polluting tanks, canals and rivers."

"This" as he added, "is a slow process"

Supervisory Control of Food and Drinks

Though, as will be discussed in the second part of this chapter the implementation of measures safeguarding the sale of foods and drinks forms an important part of any programme adopted for the suppression of cholera outbreaks it would be an unwise policy to improvise such control measures only after the disease has become manifest On the contrary in localities threatened or frequently visited by cholera, plans for regulating the sale of food and drinks during epidemics should be carefully made beforehand

[1] It is curious to note that, in contrast to this modern attitude, in the past the cholera invasions of Europe very often led to far-going improvements in public health and to institutions which proved their value in many other directions as well (Liebermeister 1896)

and those of the measures envisaged in this respect which, besides forming a safeguard against the inroads of cholera, check the spread of gastro-intestinal diseases in general ought to be constantly enforced An adequate programme for such activities has been proposed by McLaughlin (1910) in stating that

"The health officer personally or through his sanitary inspectors should exercise the closest supervision over markets, stores, restaurants hotels and other places where food and drinks are manufactured or exposed for sale. Unnecessary careless and uncleanly handling of foodstuffs should be prevented and all prepared foodstuffs protected from flies and other insects."

Public Health Propaganda and Education

The groundwork for the intensive health propaganda and education campaigns to be carried on during cholera epidemics should be laid during *times still free from manifestations of the disease* The main object of such campaigns during the off seasons is to teach the people methods of personal prophylaxis with the aid of which they can protect themselves not only against infection with *V cholerae* but also against gastro-intestinal affections in general Since however as will be discussed presently early information on the appearance of cholera is of the utmost importance for the rapid suppression of outbreaks, advantage must also be taken of the off season campaigns will be dealt with in the second part of this chapter special atten- to report immediately should they or their relatives or friends fall ill with signs suggestive of the disease

While the methods to be used for the propaganda and educational campaigns will be dealt with in the second part of this chapter special atten tion has to be drawn at the present juncture to house to-house visits which besides offering excellent opportunities for the instruction of the people through health talks, can be utilized as well to inspect the sanitary condition of the premises in question thus enabling the staff to suggest or even to carry out improvements in environmental sanitation It is regrettable that it is often difficult or even impossible to implement this eminently useful method of cholera prevention

In addition to the above-described methods, which may be said to fall largely within the scope of the general public health programme, it is necessary to make the following provisions in localities threatened or frequently visited by cholera.

Intelligence Service

To guard against the unforeseen appearance of cholera, permanent intelligence work on the part of the anti-epidemic organization is indispen sable The prime duty of this intelligence service is to keep abreast of the

possible onset of outbreaks through a constant study of the cholera situation in adjacent localities especially in areas from which groups of people such as pilgrims or seasonal labourers are apt to arrive In regions where cholera is endemic or occurs frequently due attention must be also paid to past observations on the seasonal incidence of the disease and methods of forecasting outbreaks should be used to assess the likelihood of a reappearance of the disease As a further safeguard every possible effort must be made to obtain information on incipient cholera manifestations at the earliest possible moment As will be discussed soon it is essential to enlist for this purpose the co-operation of the medical profession and in rural areas that of the village authorities as well and moreover to make arrangements for obtaining information from the owners or managers of hotels, lodging houses and similar undertakings However as noted above attempts ought to be made as well to induce the people with the aid of suitable propaganda methods to seek medical aid immediately should manifestations suspicious of cholera appear in their midst

Staff Organization

In the past it was often necessary to fight cholera outbreaks with the aid of emergency staffs hastily recruited after the infection had become rampant Nowadays this task is alleviated in that within recent times permanent public health staffs have been made available on an ever increasing scale not only in urban communities but also in rural districts or even sub-districts However invaluable as such staff groups are for some branches of the anti-epidemic work especially for keeping a constant watch for incipient cholera manifestations and trying to nip these in the bud, their size is usually too small and their duties are too manifold to make it possible for them to combat major epidemics through their own efforts. It is essential, therefore to maintain in addition to the regional public health staffs special mobile units capable of proceeding rapidly to the scene of such major outbreaks so as to take over the fight against them

The number and size of such anti-epidemic units depends upon the cholera situation in the areas in question However even if cholera shows a seasonal incidence it is desirable to keep the units at full strength throughout the year For apart from the possibility that they might thus become available for campaigns against other infectious diseases their staff may make itself most useful during cholera free times by participating in preventive activities such as the promotion of sanitary improvements, public health propaganda and education and vaccination campaigns

To be able to perform their duties efficiently the anti-epidemic units ought to be provided not only with all necessary equipment and supplies but also with sufficient motor transport

Provision for Hospitalization [1]

A further most important task of cholera preventive work is the provision of or at least the planning for sufficient hospital accommodation so that, should the disease appear prompt and adequate care can be taken of the often suddenly accumulating patients The problem of preparing for their hospitalization varies according to the local conditions In urban communities it is often possible to take advantage of existing isolation hospitals or of wards for the treatment of infectious diseases Such wards are not rarely available in rural public health stations as well, but as a rule their space is too limited to suffice during major cholera outbursts In areas frequently affected by cholera it is therefore a most recommendable practice to provide for mobile hospital units which can be rapidly shifted to the scene of such major epidemics. Benjamin (1949) who referred to the provision of three 50-bed mobile infectious diseases hospitals in Bombay State, testified to the great value of such institutions by stating that

"These hospitals have been very useful in not only reducing the case mortality but also preventing spread by contact infection in the areas where they have functioned."

Vaccination

The indications for and the efficacy of preventive vaccination against cholera will be dealt with in a separate section of this chapter

SUPPRESSIVE MEASURES

General Organization of Campaigns

Dealing with the problems of cholera control, Liebermeister (1896) maintained that

"If a cholera epidemic is present or even if it is threatening, it is most appropriate to entrust the execution of all pertinent measures to a commission composed of physicians and competent laymen, to which it is necessary to give to some extent a kind of dictatorial power The police ought to function in this work merely as an organ for implementing *the measures [Ausführungsorgan]. Among others it will be the duty of this commission* to inform the public for what reasons its orders have been issued. Regular and reliable official reports on the state of the epidemic will be instrumental in maintaining the confidence of the public, whereas attempts at hiding the truth will lead to evil consequences." [Trans.]

In view of the great progress which has been made in most countries within recent years in the codification of sanitary regulations and in the organization of public health services, there seems nowadays hardly any need for cholera commissions with the far reaching powers envisaged by

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[1] The methods for installing and running cholera hospitals will be discussed in the second part of this chapter

Liebermeister As a rule it will be preferable to leave the direction and co-ordination of the anti-epidemic activities in the hands of the senior public health officers However it may be advantageous to arrange during major cholera outbreaks, particularly in urban areas, for the functioning of advisory boards consisting of leaders of the communities in question the main task of which will be to assist the medical officers in obtaining and keeping the confidence of the population and to urge the people to co-operate in the anti-epidemic efforts

Arrangements for Detection of Patients

While the fundamental importance of the early detection of individuals attacked by cholera is generally realized opinions regarding the comparative value of the various methods available for such a "case finding" system vary considerably Many workers stress the importance of notification of all instances in which the presence of the disease appears to be manifest or may be suspected, and such notifications have been made obligatory in many countries not only for the medical personnel but also for lay authorities like for instance, the village heads in rural localities, often indeed, they are even compulsory for the heads of individual households and what is of far greater importance for the owners or managers of hotels, inns and other places where transients are apt to lodge

The usefulness of such a system of notification has been denied by other observers, who pointed out that often only those sufferers who show typically severe signs of the disease will be detected, while the numerous patients in whom the infection is manifested merely by diarrhoea, though also dangerous by the fact that they may spread the disease will remain undetected. However irrefutable as this objection is there can be no doubt that the prompt notification of typically attacked cholera patients followed by their rapid isolation will exert a great influence in reducing the spread of the infection and will at the same time be instrumental in saving the life of many of the sufferers because treatment was commenced at an early stage of the disease. The validity of this contention has been well illustrated by the following observations made according to Kamal and co-workers (1948) during the 1947 Egyptian cholera epidemic

> "Gamal-el-Din,[1] investigating cholera incidence in rural areas of Behera Province, found that in isolated farms away from the residence of mayors and which lack telephonic communications, infection was heavier than in villages where mayors live and telephones exist. Among 14 000 inhabitants in farms the incidence was 700 per 100 000 population, while among 437 000 people residing in the villages proper the rate was 240 only The sanitary conditions in the farms and the villages are the same."

However unwise though it would be to give up the system of notification of cholera attacks, it must be fully realized that the methods actually

[1] Unpublished official report

used for this purpose are often insufficient and constant endeavours must therefore be made to improve the case finding system Most important among these improvements are improved and intensified public health propaganda campaigns aiming at acquainting the people with the clinical appearances of cholera and impressing upon them that rapid isolation and treatment of the sufferers is of the utmost importance both for saving the life of the sufferers themselves and for protecting their contacts against the infection. In rural areas great attention ought to be paid as well to giving instruction regarding the recognition of cholera manifestations to the village heads or other petty officials to whom—in the absence of a trained medical staff—the reporting of cholera patients or suspects has often to be entrusted

It has been postulated by some authors that efficiency in detecting cholera patients could be greatly enhanced through a system of daily house-to-house visits throughout the affected localities. In view of the fact, however that cholera attacks usually commence suddenly at unforeseen times, frequently during the night, such daily inspections are not of so outstanding a value for the detection of cholera manifestations as it would seem at first glance. Nevertheless in view of their undeniable usefulness house to-house visits ought to be made provided that this can be done without detriment to other more essential branches of the anti-epidemic work. If however only a limited personnel is available priority ought to be given not to such routine inspections but to the assignment of staff members or preferably of squads for visits to the houses in which the presence of cholera patients or suspects has been recorded To pay attention in this way to households in which the infection has become manifest is of great importance not only in order to ascertain that proper care has been taken of the patients and their contacts and that the necessary methods of disinfection have been applied but also in order to detect, if possible, the cause of the appearance of cholera in the sufferers in question. As aptly stated in the latter connexion by Wilkinson (1943) by thus " tracing each case or cluster of cases to its source " it often becomes possible to find and to eliminate early foci of infection, for instance, by preventing the further use of contaminated well water Such efforts to approach the problem of cholera suppression from the viewpoint of etiology are not only bound to lead to gratifying immediate results, but might also be apt to contribute materially to the solution of the problem of why the manifestations of the disease arise

Careful records of all instances in which the presence of cholera in clinically suspect patients was confirmed by subsequent laboratory examination must be kept, taking the best possible advantage of graphs, so as to illustrate the trend of the outbreaks, as well as of spot maps in order to ascertain the presence of localities with massive infections calling for special attention in the anti-epidemic campaigns

Arrangements for Isolation and Management of Cholera Patients

While general agreement exists in regard to the indispensability of strict isolation of cholera patients and suspects, some of the early observers e.g., Flügge (1893) motivated by the fear that strict insistence upon hospitalization of the sufferers might lead to their being hidden raised no objection to the confinement of the patients in their homes or even recommended this However these views are no longer shared by most modern workers who stress that (1) under the conditions prevailing in the now cholera affected areas it is usually next to impossible to provide for adequate isolation of the patients in their houses , and (2) owing to the superior facilities for treatment in properly equipped and staffed institutions, hospitalization is as much in the interest of the sufferers as in that of the community Indeed, the superiority of hospital treatment is so obvious that as a rule little if any difficulty is encountered in inducing severely affected cholera patients to seek admission Certainly hospitalization would be objected to were an attempt made to bring the sufferers to far distant institutions. This, however is nowadays avoided as much as possible, because it is realized that the transport of severely cholera affected patients over long distances greatly reduces their chances of recovery and at the same time enhances the risk of spread of the infection by them

Though, as mentioned above the possibilities of setting up hospitals for the isolation of cholera patients vary according to the local conditions, attention ought to be paid to certain principles whatever the circumstances Firstly as pointed out with much reason by Flügge (1893) it is not a wise policy hastily to erect primitive barracks, should the first accommodation available for housing the sufferers prove insufficient because such temporary premises often offer no adequate shelter for the patients and are thus apt to prejudice the people against hospitalization In Flügge s opinion it was far more satisfactory therefore to install temporary cholera hospitals in existing buildings particularly in schools which had to be closed anyhow during the outbreaks and which could easily be subjected to adequate disinfection after their termination Hence, he stated,

"As a rule the installation of auxiliary lazarets, the increase of means to transport the patients, the organization of squads for disinfection and for attendance upon cholera patients kept in their houses can be taken in hand when the first local cases have occurred. Before that it is merely necessary to have carefully worked out plans for all these measures." [Trans.]

However while the policy recommended by Flügge is justified for regions where cholera visitations are not frequent, in localities where the disease appears more often or even regularly it is preferable by far to make permanent arrangements for the accommodation of the patients. As has been noted above, in urban communities there is often no lack of suitable

hospitals or wards In rural areas on the other hand, some difficulty may be encountered in this respect unless as aptly recommended by Seal (1948) for cholera-endemic areas "segregation cottages" are kept in readiness in the villages which will serve for housing initial patients pending the provision of further hospital space in schools or other suitable buildings As has been stated in this connexion, it is most desirable to have mobile hospital units in readiness to equip and staff such emergency hospitals

One of the great advantages of using permanent buildings instead of flimsy barracks for the accommodation of cholera patients is that the former are apt to be provided with solid floors and generally to be constructed in a manner facilitating disinfection should floors or walls become soiled with stools or vomits of the patients As a rule it is also less difficult to protect permanent buildings against the ingress of flies than it is to fly proof barracks. Permanent structures in rural areas such as schools or other public buildings, are usually provided with outhouses, of which advantage may be taken for the provision of fly proof morgues and of storage space

As can be gathered from the description by Higgins (1939) of an emergency hospital functioning in Shanghai during the Sino-Japanese hostilities, it appears to be possible to obtain remarkably good therapeutic results under the most primitive conditions However this and similar observations should never be taken as an excuse to neglect opportunities offering themselves for taking better care of the patients. To leave the nursing of the patients to their relatives or friends, as was necessary in the hospital observed by Higgins is particularly objectionable, and one must also deplore the practice mentioned by him of discharging the patients as soon as they could partake of rice-water Indeed one cannot help wondering whether a part of these prematurely discharged sufferers did not succumb in their homes.

Whenever possible even in temporary cholera hospitals separate provision ought to be made for (1) an admitting office for the preliminary examination of those coming for help (2) an infusion room equipped for the simultaneous administration of saline to several patients in accordance with the size of the hospital (3) separate wards for (*a*) patients who are manifestly in the acute stage of cholera, (*b*) persons suspected of suffering from the disease and (*c*) convalescents If possible separate rooms should be also provided for close observation and treatment of uraemic patients Isolation wards to take care of healthy cholera carriers may be attached

The following points for running cholera hospitals in an adequate manner deserve mention

(1) The patients brought to the admissions office ought to be examined without delay by a medical officer so as to decide whether they ought

to be hospitalized and to assign them if admitted to either the cholera or the suspects wards

(2) Either in the admissions office or as soon as they reach their ward the patients ought to be provided with hospital garments, while their own clothes are put into suitable bags for transport to the disinfection room It goes without saying that the sufferers ought also to be given bed linen and bedding by the hospital

(3) Stool samples for laboratory examination ought to be taken from both patients and suspects as soon as possible after admittance

(4) Even if fully adequate toilet facilities are available both patients and convalescents should never be permitted to utilize the toilets but should void their stools into bed pans or—in the later stage of convalescence—into the buckets of commodes, so as to permit of adequate disinfection of the stools according to the methods described in a subsequent section of the present chapter

(5) Suitable receptacles, e g., large cuspidors as used in China ought to be provided for collection of the vomits which—like the stools—must be adequately disinfected together with their containers

(6) Since soiling, not only of the garments and bedding, but also of the bedsteads the floor and the adjacent lower portions of the walls by the faeces and vomits of cholera patients in the evacuation stage of the disease is well nigh unavoidable constant care must be taken properly to disinfect these contaminated objects also

(7) Whenever possible the cholera patients ought to be attended by a trained nursing staff thus obviating any necessity for admitting their relatives and friends to the isolation hospitals which should be kept closed to all outsiders

(8) All staff members must wear overalls of the pattern generally used in wards for infectious diseases as long as they are in contact with the patients they should preferably also wear special, easily washable nether garments and rubber boots or galoshes The use of rubber gloves, which has been advocated by a few authors e g. by Aumann (1914) is by no means indispensable and is apt to prove rather inconvenient in view of the fact that as a rule cholera outbreaks run their course during hot seasons. Great care has to be taken, however to ensure that all staff members disinfect their hands whenever they have come into contact with the patients or with actually or potentially contaminated objects in the wards and that they carefully wash their hands after a thorough final disinfection when going off duty It is hardly necessary to state that the staff members should not keep food supplies nor eat in the wards and should also refrain from smoking while on duty They ought to be kept effectively immunized against cholera as Napier (1946) stated with much reason in this connexion,

hospitals or wards. In rural areas on the other hand, some difficulty may be encountered in this respect unless as aptly recommended by Seal (1948) for cholera-endemic areas, "segregation cottages" are kept in readiness in the villages, which will serve for housing initial patients pending the provision of further hospital space in schools or other suitable buildings As has been stated in this connexion it is most desirable to have mobile hospital units in readiness to equip and staff such emergency hospitals

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The following points for running cholera hospitals in an adequate manner deserve mention

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Liebermeister claimed that implementation of this measure had given good results at Basel, Switzerland, in 1855 and again at Heilbronn Germany in 1873

While he thus laid stress upon the evacuation of supposedly cholera infected houses without apparently insisting upon isolation of their inhabitants, several subsequent observers laid stress upon the segregation of the contacts of cholera patients either in their houses or in quarantine stations. Thus the adoption of this policy in Japan was announced by Takano and his colleagues (1926) Again Kolle & Prigge (1928), reiterating in a slightly modified form a statement made in 1904 by Kolle, noted that according to the Prussian law for the control of epidemic diseases, persons living in the same room or house as a cholera patient being potentially infected (*Ansteckungsverdächtige*)

" are either put under observation in the house or are taken into separate observation stations, their dejecta are examined and they are not permitted to move around with full liberty or they are not freed from medical surveillance, before their dejecta have been examined several times and have been found quite free from cholera bacteria." [Trans.]

Kamal and co-workers (1948) discussing the experiences gained during the 1947 cholera outbreak in Egypt, maintained that " isolation of household contacts is of no less importance than isolation of cases " However, they realized the difficulties of housing and feeding large numbers of contacts during major cholera outbreaks, and considered that the health authorities had " to weigh the pros and cons of this procedure according to its circumstances "

Trying to assess the validity of the statements quoted above the present writer finds it impossible to agree that there is such an urgent demand for the isolation of all contacts of cholera patients as has been postulated by some observers As has been discussed in Chapter 10 contact infection, while generally speaking not playing a preponderant role in the spread of cholera, may become rampant in premises where people live crowded together under particularly insanitary conditions Certainly failing the possibility of cutting short a direct spread of the infection in such foci through sanitary improvements one should not hesitate to resort to wholesale evacuation of the houses or compounds in question. If however as is ordinarily the case, cholera shows no tendency to spread in the affected households, it is legitimate instead of resorting to evacuation, to adopt a system of surveillance, the preliminary and at the same time most important steps of which ought to be (*a*) an adequate disinfection of the houses, especially of the rooms where the cholera patients had been confined before they had been hospitalized and (*b*) endeavours to establish why cholera appeared in the house in question and, if possible to prevent a further spread of the disease from the source of infection thus ascertained

vaccinations should preferably be administered to the staff members not later than one week before they commence their work but since one need not fear a negative phase it is not necessary to cling to this rule in emergencies.

Disposal of Cholera Victims

Provided that elementary precautions are taken against a possible spread of the disease through flies as well as against contact infection, the disposal of the dead bodies of cholera victims is hardly fraught with danger As noted before such dead bodies ought to be kept in fly proofed morgues. They ought preferably to be handled exclusively by properly protected staff members, but it would be unwise strictly to adhere to this rule if dealing with cholera victims for the burial of which special religious ceremonies are prescribed. Particular care has to be taken in this respect if one has to do with populations or groups of people belonging to the Mohammedan faith. Accordingly, as stated by Khalil (1947) the regulations for anti-cholera campaigns promulgated in Egypt in 1929 " insisted upon the absolute respect of the dead and their proper ablution and their decent burial in the presence of some of their relatives "

As Khalil added in another part of his article, the 1929 regulations prescribed that the washing of the dead bodies should be " carried out with water containing perchloride of mercury in the strength of 1/2000 " and the shroud should also be soaked in a similar solution.

Generally speaking it is a useful measure to soak the shrouds of cholera victims in an antiseptic solution of adequate concentration but it seems preferable for this to use dilutions of carbolic acid or of cresol compounds instead of mercury preparations. As an alternative to the use of antiseptics, a layer of quicklime may be put into the coffins and the dead bodies placed on this layer One should then immediately close the coffins. Elaborate funeral ceremonies, which are potentially dangerous since they lead to the assembly of many people in the foci of the infection, ought to be discouraged as much as the local customs permit

Management of Contacts

It is of historical interest to note that Liebermeister (1896) still labouring under the belief that the transmission of cholera through the air occurred " not at all rarely " insisted that

" all houses, in which cholera attacks originated and which accordingly were proved to be foci of the infection, be completely evacuated and their inhabitants be housed elsewhere throughout the epidemic and be kept under careful surveillance." [Trans.]

isolation hospitals until three consecutive examinations of their faeces had proved negative for *V. cholerae*. However as described by Kamal (1951) the routine adopted in the affected areas outside Cairo was as follows:

"From the first day of convalescence, put down as not earlier than the seventh day of the disease, a rectal swab was examined for vibrios and another three days later. If both were negative, the patient was discharged—this usually happened on or about the 12-14th day of illness. If one of the swabs was reported positive then three consecutive negative specimens had to be insisted upon, before release."

Kamal admitted that there was a discrepancy between this routine practice and observations made during the 1947 epidemic which had shown that a "non-ignorable" percentage of the convalescents still excreted cholera vibrios on the fifteenth day after onset of the disease. However it was difficult or often quite impossible to keep the patients in quarantine for more prolonged periods not only for lack of space but also because they often objected to their detention after they had clinically recovered from the disease. More important still, ample experiences during the 1947 epidemic, already alluded to in Chapter 10, had shown that a discharge of cholera convalescents not later than 15 days after onset of the disease did not lead to "return cases", i.e., secondary infections in their households.

In view of these experiences, which are in agreement with the observations made in areas frequently ravaged by cholera, it is pertinent to ask whether an elaborate system of making the discharge of the cholera patients dependent upon repeated stool examinations is really justified—the more so because in view of the frequent intermittency of vibrio excretion by the convalescents negative results obtained with occasional specimens constitute no proof that the persons in question have become really free from *V. cholerae*. However, whatever opinion one may hold in regard to the epidemiological importance of the convalescents, it is essential to keep them in the hospitals for about 10-12 days in order to give the sufferers, who often come from underprivileged strata of the population, a fair chance to regain their strength and to guard at the same time against deaths from heart failure and the late appearance of complications.

Healthy carriers

The necessity of taking precautions in the case of healthy (contact) carriers of *V. cholerae* has been stressed by several authors, almost invariably persons who gained their experience in regions other than those mainly ravaged by cholera. The most far-reaching demands in this respect have been made by Corpus (1931), a worker in the Philippine Islands, who, as summarized in the *Tropical Diseases Bulletin* (1932), expressed the opinion

"that there should be regulations obliging the entire population to submit stool specimens to enable the officials to determine the presence of carriers and there should be laws requiring the parole of cholera carriers for a period of four years."

As recommended by McLaughlin (1910), after hospitalization of the patients and disinfection of the premises the contacts should be cared for as follows

" The hands of the contacts and such clothing as may have been exposed to infection must be disinfected, and the contacts visited twice daily for a period of five days. During these five days there should be at least two examinations of the stools of each contact, one as soon as possible after discovery of the initial case and the other before discharge from observation. Should either of these examinations prove positive for cholera vibrios the contact must be isolated at once and the same precautions taken as in any other case of cholera. Until two vibrio-negative reports are received stools of contacts and their hands are to be disinfected precisely as in actual cholera cases."

Adequate as these procedures are they are so elaborate that their implementation during major cholera outbreaks will often prove impossible. There ought to be no difficulty however in implementing a minimum programme consisting of daily visits to the houses in order to detect the appearance of clinical signs of cholera among the contacts and to take prompt steps for the hospitalization of the persons concerned.

Management of Carriers

Convalescent carriers

Though, as has been discussed in the preceding chapter no convincing evidence exists that, except immediately after recovery cholera convalescents play a role in the spread of the infection, several authors, believing the contrary insisted that such individuals should not be discharged from the hospitals before their stools had repeatedly been found free from vibrios. This view was advocated for instance at an early date by McLaughlin (1910) who maintained that "convalescents should have three vibrio-negative reports of stools examined on successive days and should never be discharged upon one vibrio-negative report."

According to Crowell & Johnston (1917) an even more exacting system of examining cholera convalescents and carriers had been adopted in Manila, thus

" Cholera carriers and cases are discharged from the hospital after 3 successive negative examinations of the faeces at 2-day intervals. All cholera carriers and recovered cholera cases are followed to their houses and examined weekly for a period of two months. If they are found positive, they are returned to the hospitals."

In view of (*a*) the unsatisfactory state of the methods for laboratory diagnosis of cholera at the time the above statement was made and (*b*) the possibility of reinfections, not much weight can be given to the claim of the two authors that with the aid of their follow up system " about 27 cases were so returned in eight weeks "

As stated by Gohar & Makkawi (1948) during the 1947 cholera epidemic in Egypt it was the practice in Cairo not to discharge the patients from the

administered to carriers of *V. cholerae* in the hope of thus speeding up the disappearance of the organisms from the stools. The following of these therapeutic substances deserve special mention

Drug	*Observations*
Calomel	Found useful for the treatment of cholera carriers by some workers, e.g., by Markl (1911). However Bertarelli (1916) obtained no satisfactory results in this respect, while Jude & Millischer (1932, 1933) found the drug of little value for the "sterilization" of healthy cholera carriers as compared to oral administration of cholera vaccine.
Potassium permanganate	Recommended for the treatment of cholera carriers by observers such as França (1911), Goéré (1913) and Strikower (1913). However Creel (1912) administering the drug in combination with hexamethylenamine to a convalescent carrier of long standing, found this therapy of no use, while Heggs (1924) had no success when giving potassium permanganate as a drink to cholera carriers.
Yoghourt and lactic acid	Markl (1911) and a few subsequent workers like Bertarelli (1916) found yoghourt useful for the treatment of cholera carriers. The administration of lactic acid was recommended for the same purpose by Gilmour (1929). As will be further discussed below, Normet (1931) insisted that 5 g of the latter drug in 250 ml of water ought to be given to cholera carriers immediately before vaccination.
Carbo animale	Used (in combination with tincture of iodine) for the treatment of healthy cholera carriers by Müller (1915) and also found useful for this purpose in a few instances by Bertarelli (1916).
Hexamethylenamine	Hexamethylenamine, better known under the proprietary name of Urotropin, has been recommended for the treatment of cholera carriers on experimental grounds by Greig (1915) and has been actually used for this purpose by Johnston (1919) and, in combination with salol, by Leiva (1932). The latter recorded equally satisfactory results in the treatment of cholera carriers with heptyl resorcinol (Di-hydranol).
Chiniofon and allied preparations	Chiniofon or iodo-hydroxyquinoline, better known under the brand name of Yatren, was considered to be suitable for the treatment of cholera carriers by Kiribayashi & Aida (1932), because given in doses of 1.5 g to 2 g per day it seemed to lead to the disappearance of the vibrios in 2-5 days. However Gohar and co-workers (1952) found that administration of a similar preparation (Enterovioform) to a group of 20 healthy cholera carriers did not shorten the period of vibrio excretion in comparison with a control group (see also the tabulation overleaf).

It is not surprising to find that at present no further use is being made of the above mentioned or other drugs, such as glycerine, tannin or tincture of iodine, for the treatment of healthy cholera carriers. For in view of the fact

While these postulations which are hardly practicable and are, moreover based upon an erroneous belief in the existence of chronic carriers of *V cholerae* are merely curious, it deserves mention that in the opinion of some other observers, such as Conseil (1912) Babes (1914) Takano and colleagues (1926) Strong (1944) and Kamal (1951) it was essential during cholera epidemics to pay great attention to the detection of contact carriers and to handle such individuals in a manner similar to that adopted in the case of convalescents. Strong (1944) for instance stated in this respect that

" An important municipal measure for the control of a cholera outbreak is the diagnosing of cholera carriers such cases often occurring in those associated with a cholera case. Such carriers should be isolated and their stools disinfected until at least 2 negative examinations show them to have ceased being cholera carriers."

Kamal (1951) considered it "imperative" that contact carriers of *V cholerae* should remain isolated until three consecutive stool examinations made three days apart, had given consistently negative results

Since however as has been discussed in the preceding chapter the opinions held by the above-quoted observers regarding the possibility of healthy carriers playing a dangerous role in the spread of cholera have not been shared by workers who have had ample opportunities to study the epidemiology of the disease in India and adjacent countries with frequent outbreaks, it does not appear that search for and isolation of such carriers *are indispensable measures for the proper conduct of anti-cholera campaigns.* It is also important to realize that, owing to the intermittency of vibrio excretion by the carriers, it would be impossible to detect all of them or to establish with certainty that those who have been detained have really become free from the organisms when the time of their discharge from the isolation camps appears to have come

Under these circumstances the present writer for one is of the opinion that, even were it at all possible to search for and to isolate the contact carriers of *V cholerae* during major outbreaks, the great efforts made in this respect to the detriment of more essential work would not be justified—the less so because attempts to bring the carriers (or in order to detect them, even the contacts in general) to isolation camps would antagonize the people to such a degree that they would try to hide the appearance of cholera in their houses or would run away after the disease had become manifest in their midst.

Treatment of Cholera Carriers

Conventional drugs

In the past various drugs, considered to be efficacious for the treatment of gastro-intestinal infections and particularly cholera, have also been

following data on the response to sulfonamides of 88 convalescent carriers in Tanta

Treatment	*Number treated*	*Positive once only number*		*Positive more than once number*	
Sulfaguanidine	49	38	77 7	11	22.4
Sulfacetamide	8	2	25 0	6	75 0
No sulfonamides	31	4	12.9	27	87 0

It will be noted that as far as these figures go sulfaguanidine proved by far the more satisfactory but attention has to be paid to the fact that many more carriers received this drug than sulfacetamide.

Favourable results with sulfaguanidine were recorded by Kamal and his colleagues (1948) as follows

(1) As a rule administration of 3-4 g of sulfaguanidine four hourly for 5-7 days led to a much earlier disappearance of *V cholerae* from the stools of contact carriers than was the case in the controls

(2) There was a decided difference between the percentage of carriers among two groups of cholera convalescents treated respectively with and without sulfonamides the first group (326 convalescents) had a carrier rate of 11 6% as against a rate of 20.5 % in the 244 controls

(3) As a rule the vibrios disappeared more rapidly from the stools of 40 carriers given 4 g of sulfaguanidine for 5 days after the first positive specimen had been reported during convalescence than was the case in an equally large control group

Thus as summarized by Kamal (1951) (*a*) sulfaguanidine, given to convalescents after hydration had become complete, diminished the carrier rate among them and (*b*) administration of this drug speeded the clearance rate in cholera carriers, more markedly in contact carriers than in convalescents Therefore he postulated,

"To my mind sulpha drugs, especially the insoluble ones and maybe the new antibiotics, have a place in diminishing and speeding the clearance of the carrier state in cholera.

"The drug of choice in my opinion is sulphaguanidine and need for further experimentation on this matter on a larger and better controlled series of cases is recommended."

The observations made when treating cholera patients with antibiotics [1] render it rather doubtful whether sulfaguanidine may still be regarded as the drug of choice for the treatment of the carrier state However as far as the present writer is aware so far no trials have been made to administer the antibiotics used for cholera therapy to healthy carriers of the causative organisms

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[1] Observations on a small group of cholera patients recorded in the 1955-56 report of the Calcutta School of Tropical Medicine confirmed that treatment with tetracycline significantly shortened the period of vibrio excretion.

that such individuals frequently become spontaneously free from *V cholerae* within a few days, it seems rather questionable whether the success claimed for any of the above mentioned or other forms of treatment was not merely apparent rather than really due to the therapeutic method in question.

Sulfonamides

Administration of sulfonamides was resorted to during the 1947 Egyptian epidemic by various workers, but it is dismaying to note that they reached no agreement regarding the value of this medication for the management of cholera carriers

El Ramli (1948) treating 42 healthy carriers with various sulfonamides (18 with sulfaguanidine and 12 with sulfadiazine) declared that these drugs " proved ineffective in freeing the patients or healthy contact carriers from the cholera vibrios " while as quoted in the *Tropical Diseases Bulletin*, Wahid (1948) observed " no dramatic effect of sulphonamide prophylaxis on the carrier state " In accordance with these statements, Gohar and co-workers (1952) recorded the following results

Number treated	*Drug used*	*Dosage in grams*	*Duration of treatment (days)*	*Duration of carrier state (days)*		
				maximum	*minimum*	*average*
20	Sulfaguanidine	3×4	5-9	8	2	4
20	Sulfathalamid	2×4	5-9	7	3	4.9
20	Formocibazole	3×4	5-9	7	3	4 7
20	Sulfadiazine	1×4	5	6	2	4 1
20	Enterovioform	0 5×3	5-9	4	2	3 6
20	Atebrin	0.2×3	2	6	3	4.3
20	Atebrin	0 1×3	3	6	3	4.3
40	Controls	—	—	8	2	3 7

Thus as Gohar and his colleagues concluded, there was

" no significant difference between the treated and untreated groups and one may be justified in concluding that these substances had little or no effect on the duration of the carrier state in the contact carrier a state which without treatment persists only for a short period."

It was noted however that while during the period of observation cholera appeared in 10 of the controls, only one of the treated group, who had been given Atebrin, fell a victim to the disease Hence as Gohar and co-workers maintained, " it is not unlikely that though treatment did not shorten the duration of the carrier state it probably limited the multiplica tion of the organisms "

Favourable reports on the influence of sulfonamide treatment on the carrier state in cholera were rendered by Shousha (1948) and by Kamal and colleagues (1948) The first mentioned of these observers submitted the

Parenteral vaccination

The question whether parenteral cholera vaccination exerts an influence on the carrier state has been the subject of considerable dissension the points at issue being (*a*) whether the administration of the vaccine after the presence of *V cholerae* has been detected in the stools of contacts shortens the carrier state and (*b*) whether preliminary immunization with cholera vaccine leads to a lessened incidence of carriers.

Regarding the first problem, it was maintained by a few writers, e g. by de Raadt (1916) that parenteral cholera vaccination was a means of freeing the carriers from the vibrios but this contention was vigorously opposed by Flu (1916) who was able to point in this connexion to an earlier statement of Babes (1914) declaring that cholera vaccination " does not shorten the length of the carrier state " Agreement with the opinion of Babes and Flu was expressed by Teague (1921)

While most subsequent workers felt convinced that vaccination of healthy cholera carriers although not effective in the above mentioned sense was quite innocuous for the individuals in question Normet (1931) maintained that the vaccine administration sometimes led to the appearance of severe cholera attacks and that it was therefore necessary to avert this danger by giving the carriers 5 g of lactic acid immediately before vaccina tion. However since Normet s contention is not in agreement with the now generally accepted opinion regarding the absence of a negative phase following cholera vaccination there can be no doubt that the appearance of severe signs of the disease in some of the carriers vaccinated by him was of an accidental nature the individuals in question happening to incubate the infection at the time of the vaccine administration. There is no reason, therefore to revise the opinion of Babes (1914) that " vaccina tion of carriers of cholera bacilli is innocuous for these individuals "

The early claim of Babes (1914) that, generally speaking, the develop- ment of the carrier state was fairly rare in individuals who had been solidly vaccinated against cholera was endorsed by some subsequent authors such as Nomura and colleagues (1921) but was opposed by Pottevin & Abt (1925) who referred in this connexion to observations made during the First World War in the Italian Army showing an equally high incidence of cholera in specifically immunized and non vaccinated individuals.

In the opinion of Couvy (1933) no definite proof existed that cholera vaccinated individuals were either less or more apt to become healthy carriers of *V cholerae* However cholera vaccination was apt indirectly to exert a favourable influence on the carrier state by reducing the morbidity and consequently the number of the most dangerous incubatory carriers and of the convalescent carriers Couvy pointed in this connexion to observations of de Vogel (1925) which showed an absence of cholera

Before discussing the influence exerted on the carrier state in cholera by parenteral vaccine administration, brief mention has to be made of the following methods of treating cholera carriers which attracted but ephemeral attention.

Serum administration

It is curious to note that Salimbeni & Orticoni (1913) conceived the idea of influencing the length of the carrier state by administering to *34 healthy carriers of V cholerae 50 ml of cholera immune serum in 200 ml* of saline in the form of an enema. They found that these intrarectal serum administrations led to a disappearance of the vibrios from the stools after not later than three to six days usually within two days and that none of the carriers thus treated manifested signs of cholera. On the other hand, several individuals of a control group continued to excrete the vibrios for more prolonged periods, up to 15 days, and at least two of them became victims of the disease

Bacteriophage administration

According to Couvy s summary (1933) Doorenbos in 1931 and 1932 treated a number of cholera carriers detected at the El Tor quarantine station with bacteriophage and noted " in certain cases " a disappearance of the vibrios from the stools within 24 hours after the commencement of phage administration

Oral vaccination

Remarkable observations on the oral administration of cholera vaccine to healthy carriers of *V cholerae* have been recorded by Jude & Millischer (1932, 1933) as well as by Huri (1933) who however seems to have referred to the same group of carriers as the two first mentioned workers As these observers summarized in their 1933 report on the measures to protect Syria against an invasion of cholera from Iraq,

" The first vibrio carriers, from 30 August to 10 September 1931 were not given oral vaccination one simply administered fractionated doses of calomel to them their carrier state persisted on an average for 3 days, and in one instance only for 5 days. From 10 September onwards, the calomel was replaced by the ingestion of vaccine this consisted of a suspension, heated at 60° in normal saline of a 24-hours-old agar culture of a cholera strain from Basra the titre was 5 milliards of organisms per ml and the dose ingested amounted to 5-6 ml.

From that date, 72 cholera carriers were treated in this manner in whom stool examinations proved negative for cholera vibrios from the day following the vaccine administration." [Trans.]

for generally speaking the main attention has to be paid in this disease to *current* disinfection of the stools and vomits and of objects contaminated by these evacuations.

While other procedures, e g destruction by fire (see, for instance Knapton 1913) have occasionally been recommended to render the dejecta of cholera patients innocuous, as a rule preference is given for this purpose to the addition of disinfectants to the stools Past workers were often in favour of relying for this on sulfate of iron However Koch (1884) insisted that this chemical exerted no specific action on the cholera vibrios and was not a disinfectant in the proper sense merely stopping the growth of the organisms instead of destroying them. He emphasized that in general a clear distinction had to be made between compounds which merely counteracted putrefaction and those which were bactericidal, for it was quite possible that mere interference with the putrefactive processes was instrumental in preserving the agents of infection

Crude hydrochloric acid, recommended by Liebermeister (1896) in addition to sulfate of iron for the disinfection of cholera stools is also not used any more modern workers relying for this purpose either on one of the modern disinfectants or on milk of lime or chloride of lime

Among the usual disinfectants used for the treatment of cholera stools and deserving special mention besides simple 3% 5% solutions of carbolic acid or 1/1000 solution of mercury perchloride are (*a*) a 1/2000 solution of "sublimate" (perchloride of mercury) in saline mentioned as effective by Flügge (1893) but considered too dangerous by him for current use (*b*) carbol soap solution prepared according to the same author by dissolving 3 parts of green soap in 100 parts of hot water and adding 3-5 parts of carbolic acid (*c*) 5% solutions of the modern cresol compounds preferred at present to carbolic soap solution for the sake of expediency The procedure invariably adopted when utilizing these or similar disinfectants is to mix them in equal parts with the cholera dejecta and let the mixtures stand for one hour when the stools can be safely disposed of for instance by pouring them into the toilets or latrines

Referring to the use of milk of lime for the disinfection of cholera stools which has been recommended side by side with, or in place of disinfectants by some of the early writers Kolle (1904) stated that

"In the presence of organic substances, in putrefying mixtures and in the dejecta of cholera patients lime, in the form of milk of lime (1 part of lime to 4 l of water) has proved a potent cholera disinfectant. As Pfuhl (1892) has demonstrated destruction of the cholera vibrios in the dejecta occurs as soon as the latter have become alkaline in reaction, within one hour provided that from time to time one thoroughly stirs the mixture [of equal parts of 20 / milk of lime and the stools to be treated]." [Trans.]

In the place of milk of lime chlorinated lime in a proportion of 1 pound to 4 gallons of water may be added to equal quantities of the stools to be disinfected, or a corresponding amount of chloride of lime in powder

among the Mecca pilgrims who had left the Netherlands Indies during the 1913-14 epidemic but had been vaccinated before departure

Interesting modern observations on the relation between the carrier state in cholera and vaccination have been recorded by Gohar and colleagues (1952) who thus tabulated their findings

Time of vaccination	*Total persons*	*Number of carriers*	*Percentage of carriers*	*Duration of carrier state (days)*
On admission	233	10	4.3	4 7
1 5 days before	426	18	4.2	3 6
6-10 days before	127	4	3.1	4
More than 10 days before	76	2	2.6	3

While noting the absence of significant differences between the various groups, Gohar and co-workers pointed out that

"If anything, vaccination more than ten days before admission, i.e. a longer period before exposure to infection, may slightly diminish the carrier rate as well as the duration of the carrier state. In the light of recent knowledge about the occurrence of copro-antibodies, this is what would be expected. Thus Burrows *et al.* (1947), while carrying out an extensive investigation on immunity to Asiatic cholera, found that antibody activity measured in terms of agglutinin and protective antibody could be demonstrated in the faeces of infected and immunized guinea-pigs. Also Gohar *et al.* (1950) detected in the faeces of normal individuals low titre agglutinins which are likely to be increased by vaccination."

Disinfection

In the past, when the modes of infection in cholera were still unknown, or at least not exactly known, measures of disinfection were implemented on an often almost incredibly large scale so that, as Koch put it in 1884 millions were squandered to pour disinfectants into sewers and latrines, but after the discovery of the *V cholerae* care was concentrated "on rendering the cholera germs innocuous at the places which they *actually* reach" (Kolle 1904) This includes, besides the primarily important disinfection of the cholera infected faeces and vomits, attention to the clothes and bed linen of the patients, to a lesser extent also to the clothes worn by their contacts and to the objects used by the sufferers as well as to their beds and to the adjacent parts of floors and walls Fumigation of the sick rooms or houses with formol is unnecessary unless, as pointed out with reason by McLaughlin (1910), one has to deal with rooms containing objects or fabrics which would be ruined by the usual methods of disinfection, such as immersion into antiseptic fluids, sterilization by boiling or with steam, or exposure to dry heat.

Though, after cholera patients have been hospitalized or their dead bodies have been removed, an adequate *terminal* disinfection of the actually or potentially infected parts of the rooms or houses in question is called

Temporary Improvement of Water Supplies

The various methods adopted for the temporary improvement of water supplies at times when cholera is present or imminent fall into two main categories—namely (*a*) those which cannot be used on a large scale but merely by individuals or by individual households and thus form part of the measures for personal prophylaxis to be dealt with in a separate section of this chapter and (*b*) procedures applicable for larger groups of people The important methods falling into the latter category may be classified as follows.

Temporary supply of waterworks water

As has been discussed above, it is of the utmost importance for the purpose of cholera prevention that the water works water usually provided in cities and towns be permanently available to all inhabitants regardless of their financial status If however as is still unfortunately often the case no such permanent arrangements have been made, it is imperative that at times when cholera is present or threatening the safe water be temporarily provided to everybody The method sometimes adopted of utilizing water wagons for the latter purpose is rather unsatisfactory for various reasons principally because such a supply system, even if capable of providing a sufficiency of drinking water hardly ever furnishes enough water also to take care of all other household needs particularly of washing the cooking, eating and drinking utensils The system of temporarily installing stand pipes at suitable points of the thoroughfares is far preferable, but it must be realized that it gives fully satisfactory results only if the number of these supply points is sufficiently large to give the people no excuse to continue in part with the use of the unpurified water supplies upon which they pre viously relied For the same reason it is a most obnoxious practice to furnish water from the standpipes only at certain times instead of making it freely available to the people at all times. The situation is still worsened if as is occasionally done payment is exacted for the water supplied from the standpipes or from the water wagons

Installation of temporary water purification plants

Occasional attempts have been made at the time of cholera epidemics in communities not provided with waterworks to install some kind of tem porary plant for purification of the water supplies For instance special attention was paid to this possibility by Robertson & Pollitzer (1939) and by Pollitzer (1948) because they were confronted by the difficult task of combating cholera outbreaks in the Hunan Province of China due mainly to the wholesale consumption of contaminated river water As Pollitzer stated, an attempt was sometimes made to provide temporary sand filtration

form may be incorporated into the dejecta through constant stirring As described by Greig (1913) the systematic use of this cheap and simple method of stool disinfection proved rapidly effective during a cholera outbreak in Puri town, the more so as the pungent order of the compound was apt to keep the flies away from the dejecta. The great value of chloride of lime for the suppression of cholera manifestations was again stressed by Duggal (1949) according to whom

"The only reliable method of controlling Cholera appears to be by destroying the organism wherever it is likely to occur by the use of bleaching powder which is the par excellence disinfectant for this purpose. Water supplies should be periodically disinfected by the breaking point method and its use should be made for disinfecting excreta etc. This method of controlling is practical and simple and can be carried out without skilled personnel. In contrast inoculation is difficult to carry out in big populations satisfactorily and requires large numbers of skilled workers and much equipment and organisation."

Depending upon their nature and the available facilities, various methods have to be used for the disinfection of objects which have become contaminated with cholera stools or vomits. Liebermeister (1896) aptly stated in this connexion that

" It is best to burn objects of little value. The next best means of disinfection is boiling in water or, in the case of objects which cannot be boiled, sufficient treatment with moist steam of at least 100°C. Disinfection with hot air or with superheated dry steam is less reliable because the high temperature does not penetrate into all parts. A suitable disinfecting apparatus, in which also larger objects and particularly mattresses can be treated with hot steam, is anyhow urgently needed in every hospital and transportable apparatuses for disinfection are available. Dirty water and bath-water can be disinfected through addition of sufficient milk of lime to produce a strongly alkaline reaction, or with the aid of chlorinated lime." [Trans.]

Contrary to the advice of Liebermeister it is often recommended that the contaminated body and bed-linen of cholera patients be immersed in disinfecting fluids, but there can be no doubt that it is more reliable and at the same time far more expedient to use for such articles, which as a rule have to be washed after they have been disinfected, the methods of boiling or steam sterilization. The latter procedure is the method of choice for dealing with the clothes of patients, but since during the usual summer outbreaks of cholera in South-East and East Asia the sufferers are generally clad in light garments there is as a rule no difficulty in boiling or immersing in disinfectants in the case of their clothes as well as in that of their linen.

The above-described carbol soap solution or a 2.5% solution of one of the modern cresol compounds may be utilized for mopping or washing contaminated parts of the patients beds and adjacent portions of the floors and walls. However equally reliable results may be obtained with chlorinated lime solutions of the same strength as prescribed for stool disinfection. Alternatively chlorinated lime in powder form may be sprinkled on contaminated spots on the floors.

Environmental sanitation at and around water-collecting stations

It is hardly necessary to state that during cholera outbreaks the greatest attention must be paid to sanitation of such sources of water supply as shallow wells, tanks, ponds and rivers. Every possible endeavour must be made to prevent their pollution with faeces, as may happen through voiding the contents of commodes or night soil buckets into the tanks, ponds or rivers or washing these containers in their water At the same time it is essential to provide as much as possible for sanitation of the surroundings of such water-collecting stations. A programme of general cleanliness ought to be enforced Whenever feasible, insanitary latrines in the vicinity should be closed and faulty sewers should be improved Refuse dumps situated nearby must be removed. In order to obtain comparatively pure water supplies away from the shores of rivers or ponds the construction of floating platforms was found to be advantageous (Robertson & Pollitzer 1939)

Prohibition of use of dangerous water supplies

Theoretically it would no doubt be best at the time of cholera outbreaks to prohibit the use of the water from wells tanks or ponds and rivers which appear to be dangerous for the spread of the infection In actual practice, however it is often impossible to adopt this drastic method, both because —except in the case of wells the water of which can be made unpalatable through the addition of excessive amounts of chemicals such as potassium permanganate—it is rather difficult to enforce and, more important still, because it is frequently not possible to make rapid provision for sufficiently abundant and otherwise satisfactory substitute sources of water supply Under these circumstances it is fortunate that, as will now be described simple, yet quite reliable methods for disinfection of the water supplies are available.

Water disinfection

Depending upon the circumstances various methods have been used to disinfect cholera-contaminated water supplies Thus Koch (1893) referring to the waterworks system in Nietleben, stated that

"The disinfection of the system [*Leitung*] did not cause unduly great difficulties. One could have used for it diluted milk of lime, carbol solution or a mineral acid. Carbolic acid was chosen, a 3 / solution of which was driven from the intake into all parts of the system, left to act for 24 hours and then washed away with Halle waterworks water One is entitled to assume that in this manner reliable disinfection was effected. A drawback of the method was that the waterworks water had an unpleasant taste of carbolic acid for a fairly long time. However in comparison with the two other disinfectants carbolic acid had the advantage of not leading to obstruction of the pipes, as was feared in the case of

stations at the places on the river shores frequented for the purpose of water collection, it being made incumbent on the water carriers to pour raw water on the sand filters with the aid of special containers and then to fill their own buckets with filtered water However the difficulties of providing sufficiently large filter plants and of ensuring their proper use were as a rule so overwhelming that, as will be described below it was found preferable to adopt a system of adding chlorine solution to the water in the buckets filled by the carriers

As described by Robertson & Pollitzer a still more ambitious scheme was

" the construction of a floating barge anchored out in the river and accessible by a gang way The barge naturally rose and fell with fluctuations of the river water level. Water was admitted to a compartment by gravity the barge being suitably ballasted. The river water passed through compartments where it was filtered and then the proper amount of chlorine solution added."

Unfortunately owing to an error in calculation as to the weight of the ballast, this barge sank soon after it had been installed. However, in another town successful, though limited, advantage could be taken of a smaller barge provided with a sand filtration device.

Though it must be admitted that the barges used in China did not prove outstandingly successful the present writer cannot help feeling that good advantage might be taken of the large and strongly constructed floating water purification plants provided by some firms for service in localities possessing no or no proper water supplies of their own In his opinion the availability of such barges for service in the villages situated on the shores or on the islands of the rivers in the endemic areas of India and East Pakistan might go a long way to controlling cholera manifestations which at present are most difficult to combat owing to the almost over whelming difficulties of procuring safe water supplies *in loco*

Provision of drinking-water fountains

To guard against the possibility of workers or travellers contracting infection away from their homes it is essential during cholera epidemics in communities without waterworks to place containers with boiled water at strategic points on the public thoroughfares or at other places passed by many people e g. important railway or bus stations and ferry landings This measure proved particularly beneficial in China, where the people, who usually restricted themselves to the consumption of tea or hot water when at home, quite often became cholera infected when partaking of contaminated water or other cold drinks away from their houses. As a rule there was no difficulty in interesting charitable societies or benevolent persons such as the owners of big business establishments, in the installation and the management of such drinking water stations

contents of the water, 8 ounces (about 225 g) or even more had to be used when dealing with "very foul" wells

An alternative practice was to treat one of the several wells in cholera affected communities with an excess of potassium permanganate and immediately to pump out the water until the red colour had nearly vanished The water could then be used until that of the other wells, disinfected in the usual manner was fit for consumption on the following day

An additional recommendation of Hankin s was that in cantonments the disinfection with potassium permanganate be followed by pouring into the wells an equal quantity of hydrochloric acid because this greatly increased the activity of the permanganate

As shown by ample experiences in the North West Provinces and Oudh it was necessary to repeat the well treatment with permanganate of potash every three or four days as long as the danger of cholera persisted.

Dealing with his own observations Hankin stated that out of 50 cholera outbreaks, during which well disinfection with potassium permanganate had been done under particularly careful supervision no success was obtained in 11 instances, obviously because only a portion of the water supply had been treated On the other hand fully satisfactory results were obtained in 36 epidemics in 16 of which no further attacks were reported after treatment of the wells, while in 10 outbreaks attacks occurred only within three days after well disinfection In 10 outbreaks "later cases" were observed but these

"were few in number generally occurring on the fourth or fifth day after treatment of the wells In one village it was noted that of the two later cases, one was that of a man who had refused his well to be treated"

Good results with potassium permanganate disinfection of wells were also obtained during the 1896-97 famine in the North West Provinces and Oudh when the method "undoubtedly proved a most useful agent in checking disease"

Concluding his article Hankin appropriately remarked that

"The method is of interest from the scientific standpoint, as its success appears to give proof of the truth of the view that cholera is in most cases a water borne disease."

The method advocated by Hankin was again recommended by Dunn (1913) who maintained that 1 ounce of potassium permanganate per 2000 gallons of water was sufficient for the disinfection of ordinary wells and by Maddock (1915) in whose opinion the "pinking" of cholera suspect wells had been found to be of considerable efficacy Rogers (1921) though admitting that much evidence had accumulated regarding the great value of permanganate of potash in the disinfection of wells was inclined to believe that this chemical acted rather by precipitating all organic matter in suspension than by actually killing the cholera vibrios

milk of lime, and also did not damage the interior of the pipes, as mineral acids might have done.[1]" [Trans.]

As alluded to before confronted by the difficult task of dealing with the water of a cholera-contaminated river which was largely used for house hold and even for drinking purposes, Robertson & Pollitzer (1939) adopted the system of adding a solution of chlorinated lime to each of the buckets used by the water-carriers for bringing the supplies to the houses For this purpose as the two authors described,

"Sanitary attendants were stationed at convenient points controlling the principal water collecting stations. They were provided with large earthenware vessels containing 0.25 per cent chlorine solution and with measuring cups made of bamboo with long handles. The concentration of chlorine thus added to the buckets was 0.9 p.p.m."

As the authors added,

"Chlorination of the water buckets being only one of various anti-cholera measures adopted, it is difficult to judge upon its efficacy It should also be kept in mind that this procedure could take care only of the principal water collecting stations, but not of places of lesser importance nor of the houses along the river front. Nevertheless we can say that the introduction of this system was usually followed by a considerable drop in the incidence of cholera cases sometimes the outbreaks continued in endemic rather than epidemic form."

Useful as the above methods of water disinfection occasionally are they are of little importance for the control of cholera as compared to the disinfection of wells which has been done almost exclusively by treatment with either potassium permanganate or chlorinated lime

Well disinfection with potassium permanganate

Dealing with important observations on the usefulness of well disinfec tion with permanganate of potash, Hankin (1898) pointed out

"that the method of purifying water by means of permanganate to which I have been standing as godfather is by no means new Permanganate was used in drinking water during the London cholera epidemic of 1866. Its use has been advocated by many sanitary authorities before and since, such as Parkes and Surgeon-Colonel King, the present Sanitary Commissioner of Madras. I am under the impression that it was used more especially with the object of freeing water from organic matter and that when it was proved that cholera was not due to organic matter as such, but to a specific microbe, the use of permanganate to a great extent went out of fashion, until attention was again drawn to the subject by a paper that I published in November 1894 in the *Indian Medical Gazette* and in a paper read in the following month before the Indian Medical Congress."

Hankin s practice was to add to the wells to be treated for the purpose of cholera control sufficient potassium permanganate to produce a pink colour lasting until the following day Generally 2 3 ounces (about 55-85 g) sufficed per well but, the necessary quantities varying with the organic

[1] In spite of Koch s misgivings, dilute mineral acids were afterwards successfully used for the disinfection of the pipes of waterworks systems

contents of the water 8 ounces (about 225 g) or even more had to be used when dealing with "very foul" wells

An alternative practice was to treat one of the several wells in cholera affected communities with an excess of potassium permanganate and immediately to pump out the water until the red colour had nearly vanished The water could then be used until that of the other wells disinfected in the usual manner, was fit for consumption on the following day

An additional recommendation of Hankin s was that in cantonments the disinfection with potassium permanganate be followed by pouring into the wells an equal quantity of hydrochloric acid because this greatly increased the activity of the permanganate

As shown by ample experiences in the North West Provinces and Oudh it was necessary to repeat the well treatment with permanganate of potash every three or four days as long as the danger of cholera persisted.

Dealing with his own observations Hankin stated that out of 50 cholera outbreaks, during which well disinfection with potassium permanganate had been done under particularly careful supervision, no success was obtained in 11 instances obviously because only a portion of the water supply had been treated On the other hand, fully satisfactory results were obtained in 36 epidemics, in 16 of which no further attacks were reported after treatment of the wells while in 10 outbreaks attacks occurred only within three days after well disinfection In 10 outbreaks "later cases" were observed but these

"were few in number generally occurring on the fourth or fifth day after treatment of the wells. In one village it was noted that of the two later cases, one was that of a man who had refused his well to be treated"

Good results with potassium permanganate disinfection of wells were also obtained during the 1896-97 famine in the North West Provinces and Oudh when the method "undoubtedly proved a most useful agent in checking disease"

Concluding his article Hankin appropriately remarked that

"The method is of interest from the scientific standpoint, as its success appears to give proof of the truth of the view that cholera is in most cases a water-borne disease."

The method advocated by Hankin was again recommended by Dunn (1913) who maintained that 1 ounce of potassium permanganate per 2000 gallons of water was sufficient for the disinfection of ordinary wells, and by Maddock (1915) in whose opinion the "pinking" of cholera suspect wells had been found to be of considerable efficacy Rogers (1921) though admitting that much evidence had accumulated regarding the great value of permanganate of potash in the disinfection of wells was inclined to believe that this chemical acted rather by precipitating all organic matter in suspension than by actually killing the cholera vibrios

as the amount commonly used was insufficient for the latter purpose. Dealing with the method of applying potassium permanganate Rogers stated that

"The usual allowance is one or two ounces for an ordinary well used in the following way The salt is placed in a bucket or other convenient vessel and lowered gently into the water to fill it It is then drawn up and the water poured carefully into the well without allowing the undissolved crystals to escape. This process is repeated until the whole has passed into solution when the well water should have a faint pink colour which will disappear in a day or two no harm will result from its being drunk at once."

Most modern writers have professed more or less scepticism or have even doubted that potassium permanganate disinfection of wells is an effective method of cholera control. Thus Strong (1944), agreeing with Rogers that the chemical was not vibriocidal, but merely precipitated the organic matters, considered its use for the treatment of wells less satis factory than chlorination. Frank disapproval of the method was expressed by Bhaskaran and colleagues (1944) who stated that

"there is no quick method of testing the efficacy of disinfection by potassium permanga nate in the field. Other disadvantages in the use of permanganate, viz., high cost, slow action, increase in colour fishy taste and the undesirable concentration of the residual manganese have long been recognized. For these reasons potassium permanganate is not used nowadays in countries other than India for disinfecting water in the field."

Napier (1946) was of the opinion that the simplicity of application of potassium permanganate greatly facilitated well disinfection, but pointed out that a dilution of the chemical of 1 in 500 000 (obtained by adding $\frac{1}{4}$ grain of permanganate to each gallon of water) while readily killing cholera vibrios in a short time, did not kill all coliform organisms in 24 hours

Views similar to those of Napier were propounded by Banerjea (1950), who concluded from exhaustive studies that

"In the disinfection of well waters with potassium permanganate, unless the reagent is used in a very strong solution of 1 in 50 000 producing an objectionable deep pink colour it cannot be depended upon to produce a water which may be called satisfactory according to water bacteriological standards, though 1 in 200,000 dilution might kill the number of pathogenic organisms, specially cholera vibrio, that is ordinarily likely to infect well waters. With a 1 in 50 000 strength satisfactory water could not be produced in 100 per cent of well waters, though in a large majority of them the strength will succeed after 24 hours contact."

Writing in 1952, Rogers again stressed the value of potassium perman ganate treatment of wells for the suppression of cholera outbreaks, but admitted that, while this chemical was too expensive for the disinfection of large tanks, chlorination of these as well as of wells with the aid of bleaching powder or preferably with electrolytic chlorine was cheaper and at the same time very effective.

Chlorination of wells

As far as the present writer is aware, reference to the method of chlorinating wells for the purpose of cholera control was first made by Bishop (1912, 1913) Describing the techniques he had adopted for this work in greater detail in his second paper, Bishop stated that

"Where the necessity is indicated (and this is in practically every case) purification of wells is undertaken, the agent employed being nascent chlorine obtained from the hypochlorite of calcium in commercial bleaching powder A solution of this substance of a strength to give five parts of free chlorine to a million parts of water is put into a graduated bottle, each graduation supplying a chlorine equivalent of one foot s depth of water in the well to be treated."

As the author added,

"The result of our experience of a chlorine efficiency may be conveniently stated by the formula

$$X = 15.7\ R^2 D$$

Where X = Grains of bleaching powder required.
R = radius of the well in feet.
D = depth of water in feet.

"In other words, the square of the radius in feet multiplied by the depth in feet multiplied by 15 7 gives the number of grains of bleaching powder required for the effective disinfection of any given well."

At times when cholera was prevalent, attention was also given to the treatment of tanks, which consisted "in mixing quantities of bleaching powder into the water at the places frequented by the people and is effective by rendering the water at these places unpalatable"

Effective as this method undoubtedly is, it should be implemented only if alternative and sufficiently abundant sources of water can be made available to the people. Even then such superchlorination may be unwise, since it is likely to lend strength to the prejudices of the people against the usual method of well chlorination which they sometimes claim, renders the water harmful for consumption (see, for instance, Robertson & Pollitzer 1939) It is preferable, therefore to use superchlorination only if the excess of chlorine can be removed with the aid of sodium thiosulfate or by other means (see Traube 1894 and Harding, 1910)

Numerous subsequent observers were unanimous in asserting the great value of well chlorination for the suppression of cholera outbreaks. Thus, to quote an example Duggal (1949) referring to the observations he had been able to make in Bihar, stated that

"At first frequent disinfections of water supplies were started in selected areas of civil population adjoining army camps during the period of the Great World War II. It was noticed that this practice affected the incidence of Cholera significantly Such areas remained free from outbreaks of epidemics while the adjoining civil areas where the well water supplies were not treated with bleaching powder were visited by seasonal outbreaks of Cholera. The wells are disinfected to breaking point by bleaching powder so that

the chlorine smell is noticeable to the consumers. Disinfection of water supplies preferably every week during Cholera season is practised but in far off distant places in rural areas it is done at greater intervals. In infected areas the open wells are disinfected twice a week."

It is unfortunate however that so far no agreement has been reached regarding the dosages of bleaching powder to be used for well disinfection. Several writers consider a residual chlorine content varying from not less than 4/10ths part per million of water (see for instance, Shattuck, 1951) up to the often mentioned figure of 1 part per million to be satisfactory but others refer to lesser or higher standards Henderson & Seneca (1951) for instance speaking of the necessity of producing "a residual chlorine content of 2 ppm. or higher" Certainly as Napier (1946) put it,

"as the chlorine content of bleaching powder varies from sample to sample, and as the chlorine fixing power of different water supplies also varies, no rule of thumb can be adopted and the amount to be added must be calculated for each well or cistern"

Napier described three standard solutions to be prepared for this purpose—namely (*a*) a 1/1000 solution of the bleaching powder to be used (*b*) 10% potassium iodide solution and (*c*) 1% starch solution.

Before making the test, the volume of the well to be treated was calculated from the depth of the water and the radius of the well by using the formula $\pi r^2 \times$ depth, according to which for instance a well 10 feet deep and with a diameter of 6 feet had a capacity of 1768 gallons. The cubic content of tanks and cisterns was ascertained by multiplying their length by their breadth by the depth of the water bearing in mind that one cubic foot of water was equivalent to $6\frac{1}{4}$ gallons.

As Napier continued the actual standard test was carried out in the following manner

"Take five white bowls or flasks and in each place 500 c.cm. of water to be treated.

"Take a clean graduated 1 c.cm. pipette, and wash it thoroughly with distilled water With this pipette add varying amounts of the 1 in 1 000 bleaching powder solution to the water in the five vessels, 0.5 c.cm. 0 7 c.cm. 0 9 c.cm., 1 1 c.cm. and 1.2 c.cm. to the first, second, third, fourth and fifth bowl respectively

"Stir the mixture in each bowl with a clean glass rod, beginning with the bowl containing the least amount of chlorine solution, and going to the one containing the next smallest, and so on.

"Allow them to stand for at least an hour Then test for free chlorine by adding to each bowl about 1 c.cm. of 10 per cent potassium iodide solution and 1 c.cm. of freshly prepared starch solution. Mix well and note the first bowl that gives a faint blue colour Note the amount of bleaching powder solution that was added to that particular bowl and multiply by 20 The result gives the number of pounds of bleaching powder required for one million gallons of water to this figure add 3 pounds which, if the bleaching powder is approximately 30 per cent, is the usual safety margin allowed for one million gallons of water Now calculate the amount of bleaching powder that should be added for the amount of water already ascertained to be present in the well or cistern that is to be chlorinated."

For instance, if the third bowl was the first to give a faint blue colour then $0.9 \times 20 = 18$ pounds, so that, adding 3 pounds, 21 pounds of

bleaching powder had to be used per million gallons of water In the case of the above mentioned well containing 1768 gallons of water the amount of bleaching powder required would be

$$\frac{1768 \times (18 + 3) \text{ lbs}}{1\,000\,000} = 0\,037128 \text{ lb} = 260 \text{ grains or about 17 grams}$$

As added by Napier a rough alternative method was to place a pint of water into each of the above mentioned bowls, and to add 10 15 20, 25 and 30 drops respectively of the bleaching powder solution from a dropper to stir thoroughly and after at least one hour to add the potassium iodide and starch solutions Calculations are then made as follows

$$\frac{\text{Minimum number of drops of bleaching powder solution added to the first bowl in which blue colour was distinct}}{\text{Number of drops from the dropper that make a dram}} \times 0.44 + 0.021 = \text{Grains per gallon of water to be treated.}$$

For instance if the fourth bowl was the first to give a blue colour then $25/80 \times 0\,44 + 0\,021 = 0\,1585$ grains per gallon or 158 grains per 1000 gallons

Bhaskaran and co-workers (1944) making comparative studies of the sensitivity of rough tests made respectively with starch iodide solution and with orthotolidine solution in order to determine residual chlorine in water found that the tests with the former solution were less sensitive and apt to lead to overdosing with bleaching powder to the extent of 0.2 p p m. They therefore recommended the use of tests with orthotolidine, through which the extent of overdosing with bleaching powder could be considerably reduced.[1]

However though the problem as to how carry out well chlorination to the best advantage does not yet seem to be fully solved, there can be no doubt regarding the fundamental importance of this method in the control of cholera manifestations [2]

Food Control

As will be discussed when dealing below with the problems of personal prophylaxis even in the midst of a most severe cholera epidemic careful adherence to dietary rules is apt to prove a never failing means of avoiding infection. It is one of the most important objects of public health educa

[1] A description of the tests for residual chlorine with the aid of orthotolidine will be found in standard textbooks—e g., American Public Health Association (1946) *Standard methods for the examination of water and sewage* 9th ed., New York, p. 97, and Gradwohl, R. B. H. (1956) *Clinical laboratory methods and diagnosis*, 5th ed., St. Louis, Mo vol 2, p. 1672.

[2] F H Harrington and J N Lamois, in an unpublished report on the 1946 cholera outbreak in Canton stated that chlorination of the shallow wells of that city was impossible due to ground water movements which washed away the disinfectant solution applied and that consequently it was necessary to resort to chlorination of the well water after it had been collected in buckets. Certainly however difficulties of this kind are exceptional and therefore do not militate against the generally great value of well chlorination.

tion and propaganda, to which also attention will be paid below to bring this truth home to the widest possible strata of the population However since not only a lack of facilities for such work (which, indeed, can accomplish but little during the fast moving tragedy of a cholera outburst) but also popular indifference are apt to form stumbling blocks, it is as a rule necessary during cholera epidemics to take administrative action with the double aim of promoting the consumption of safe foods and of prohibiting the sale of dangerous articles

It is historically interesting that such action has been taken not only within recent years but also in past times when as described by Sticker (1912) occasional attempts were made during cholera outbreaks to enforce the use of a prescribed diet by the people (*zwangsweise Volksdiät*) The regulations made in this respect by Frank (1875) during the 1873 cholera epidemic in Munich aimed merely at the prevention of excesses of all kinds However Sticker continued, during the 1892 outbreak at Hamburg

" Elaborate dietary regulations based upon laboratory experiments were promulgated, which embarrassed even the well-to-do people. Cheese, butter fish, caviar were prohibited vegetables and fruits could be neither imported nor be taken out of the city Essentially boiled beef and boiled milk were the only permitted food articles, while practically all that the poor people were wont to eat was prohibited. Some people found it preferable to starvation to risk death from cholera or punishment by the police through consumption of the prohibited foodstuffs." [Trans.]

Even more stringent regulations were made during the 1910 and 1911 cholera outbreaks in Italy when, according to Sticker

" In many places the whole harvest of fruits and vegetables was confiscated by the sanitary police, innumerable market baskets were thrown into the sea because they contained unripe or " contaminated " fruits, whole consignments of fruits and vegetables which were destined for export to foreign countries were made unfit with carbol or lysol solutions or were sent back and used for the manufacture of manure." [Trans.]

Though no modern cholera worker would approve of the harshness with which the above measures were enforced general agreement continues to exist that during epidemics the use of freshly prepared hot food only should be strictly recommended, while the consumption of raw dishes of all kinds should be prohibited or at least discouraged. In accordance with this policy to which further reference will be made in the section on personal prophylaxis, the promulgation of the following regulations during cholera outbreaks is indicated

(1) The sale of cold foods, particularly of such articles as raw fish salads jellies ice-cream and cut fruits, should be prohibited and it is desirable to enforce the same rule in the case of candy sweetmeats and the like, which are apt to prove attractive to flies, unless they are kept protected against the access of these insects.

(2) Careful supervision should be exerted over food markets, foodshops and eating-places, not only to see that the above rules are followed, but also

in order to make the best possible arrangements for keeping these establishments in a sanitary condition particularly free from flies.

A policy found useful in this work in China was to affix large posters in all eating places advising the people to partake only of freshly prepared hot foods and of hot water or tea. The guests were also urged by these posters to ask for vessels with boiling hot water to clean their eating and drinking utensils immediately before the meals The owners of the establishments were obliged to provide this facility free of charge.

Control of Artificial Drinks

With the exception of bottled beverages prepared by firms which can be relied upon to use safe water for the manufacture of their products, the sale of artificial drinks, particularly that of lemonades and the like prepared and sold by street hawkers or on street-stands, ought to be strictly prohibited during cholera outbreaks. Whenever conditions permit, the people ought to be urged to assuage their thirst with hot tea or hot water instead of consuming cold drinks. However since people working away from their homes are often not able to frequent the tea or coffee-shops usually available in the cholera-affected localities it is—as mentioned above—essential during outbreaks to install fountains with safe drinking-water at suitable points of the public thoroughfares.

Fly Control

Though, as will be discussed presently successful use has been made within recent years of insecticides, and particularly of DDT for combating the flies during cholera outbreaks the greatest attention has to be paid to the dictum of Rozeboom (1956) that

> Regardless of the effectiveness of various insecticides in reducing fly densities, and even in reduction of enteric infections experienced observers have been re-emphasizing the importance of *sanitation* as the real solution to the fly problem."

As maintained by Rozeboom, in urban communities the most necessary measure to be adopted in this respect is the elimination of fly breeding in garbage dumps, achieved by a proper system of garbage disposal, but of great importance also are a proper system of disposal of human faeces including if possible the elimination of privies and the provision of an adequate system of garbage collection in the houses For fly control in rural areas proper manure disposal is also essential but difficult to achieve, because ordinarily the manure, since it serves as fertilizer cannot be destroyed but must be kept often without being properly protected against the access of

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"2. *Spraying* with a 10 / solution of DDT in kerosene The spraying was carried out by C/47 aircraft.

"3 *Jeep-fogging* with a 7% DDT solution in Malariol.

"4 *Spraying* DDT solution by hand-guns and mechanical pumps inside houses and establishments.

"5 "*Flitting*" cars, buses and other vehicles at certain road-block stations."

Shousha added that an evaluation of the effectiveness of DDT distribu tion by B T 13 aircraft for the control of flies was undertaken by the Insect Eradication Section of the Ministry of Public Health from which however he was able to quote only the following results

"1 There was a general decrease in the fly-population in the treated villages as compared with the control village.

"2. The frequency of spraying had little effect on the ultimate reduction of the fly population.

"3 The effect of the spray on the flies was not apparent until approximately 45 minutes after treatment.

"4 In open spaces, a kill of 72 / of all flies exposed was obtained.

"5 In narrow lanes and alleys, a kill of 24 4% of all flies exposed was obtained.

"6. In shops and houses, a kill of 44 / of all flies exposed was obtained.

"7 48 / of all flies exposed were killed."

Whether in view of these generally rather mediocre results the distribu tion of DDT by aircraft is to be recommended in cholera outbreaks seems questionable Certainly the orthodox methods of spraying appear to be preferable for treating houses and the same holds still more forcibly true in regard to the lanes and alleys which are apt to form hotbeds of the infection However, while if DDT is available it should be adequately applied to these and other danger spots as well as to the houses, one should never lose sight of the necessity for and the efficacy of, emergency methods for the improvement of environmental sanitation

Personal Prophylaxis

However difficult it may be adequately to conduct anti-cholera cam paigns on a community wide scale, even in the midst of a raging epidemic " the individual and the family group can accomplish much in protecting themselves against cholera by strict attention to the hygiene of their imme diate environment " (Siler 1944)

Dealing with the various steps to be taken in this respect, Liebermeister (1896) stated with his usual felicity that besides punctillious cleanliness the following measures of personal prophylaxis were essential

Drinking-water which appears even remotely suspicious ought to be consumed only when boiled and ought to be used in the same way for cleaning the eating utensils. One must also avoid unboiled milk and similarly ice the origin of which is not exactly known. Genuine mineral waters on the contrary may be consumed All foods must

flies. However, as described by Rozeboom, some reduction of fly breeding is obtainable through the use of larvicides He stated in this connexion that

"Those used have included hellebore at the rate of 0.5 pound per 8 bushels of manure, and powdered borax at the rate of 1 pound to 16 cubic feet An emulsion containing 2 to 4 per cent chlordane, applied at the rate of 50 to 100 mg. of the toxicant per square foot of surface, is said to give fairly good results. Good kill of larvae has been obtained through the use of 20 gm. paradichlorobenzene or 25 ml. orthodichlorobenzene per square foot of surface. If repeated in two to four days, these dosages may be reduced respectively to 15 gm. or ml. These materials are also good ovicides. Larvicides may also be applied to latrines, garbage and cadavers."

Methods of screening used either to prevent the ingress of flies into the houses or to protect food and household water supplies against these insects, if rigidly applied, are of value for the protection of the families in question against cholera, but these means of warding off the infection are rarely implemented in the households in which the disease is most likely to appear Under these circumstances and also on account of the still deplorably large number of communities in which flies are not controlled through proper measures of general sanitation, the use of insecticides during cholera outbreaks often becomes indicated.

Though practically all of the chlorinated hydrocarbons used recently for the purpose of insect control have been found effective against flies, so far most ample use to combat these insects during cholera outbreaks has been made of DDT which if applied to surfaces at the rate of 200 mg per square foot, remains toxic for these insects for three months or even longer (Rozeboom, 1956)[1] Simple recommendations made by Scudder (1949) for fly control with DDT were to use it in a concentration of 2.5% for spraying rough unpainted surfaces, in a concentration of 5% for painted or plaster surfaces and of 7 5% to treat smoothly finished surfaces in restaurants and houses the surfaces in question to be always thoroughly wetted without "run-off" For applications outside the houses, Scudder considered wettable DDT compounds in 5% sprays preferable to solutions, because likely to prove less harmful to plants Specially trained squads have to be employed both for this work and for applying DDT inside the houses to ensure its adequate use and to avoid in particular the exposure of food, eating and drinking utensils and household water supplies to the insecticide

Whereas so far the usual method of residual spraying with DDT has been resorted to for the purpose of cholera control in China and India, according to Shousha (1948) various methods of application were tried during the 1947 cholera outbreak in Egypt—namely

"1 *Fogging* with a 20 / solution of DDT in Velsicol. The fogging was done by B T 13 aircraft.

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[1] It has been established that prolonged use of DDT or of other insecticides, by killing off susceptible flies, is apt to lead to the prevalence of insecticide-fast strains. As a rule, however this drawback will not interfere with the effective use of DDT during cholera outbreaks.

Subsequent workers were unanimous in supporting Liebermeister's advice that during cholera outbreaks it is wisest to rely solely on the consumption of boiled or cooked foods, preferably partaking of the meals while they are still hot, i.e. before they could have become contaminated through flies or in other ways. Most observers held in this connexion that the method of rendering possibly contaminated fruits or vegetables innocuous for consumption in the raw state by treating them with potassium permanganate solutions, though popular, was by no means reliable. However, it was pointed out by several writers, thick-skinned fruits like bananas or oranges, could be made safe for consumption during cholera epidemics by steeping them before peeling for 2-4 minutes into boiling hot water.

A point not expressly mentioned by Liebermeister but stressed by several subsequent authors was the importance of carefully washing the hands before partaking of meals during cholera outbreaks.[1]

Universal agreement has been expressed with Liebermeister's recommendation that during cholera epidemics the drinking water must be boiled unless there is no reason whatsoever to doubt its safety. As has been noted above in this connexion, such doubt may arise even if waterworks water is available, not so much owing to defects in the central plants as on account of localized peripheral contaminations. Obviously it is safest to use boiled drinking water and also tea as soon as they have cooled down sufficiently for consumption; failing that, they must be carefully protected against contamination through flies or by other means.

The methods recommended for rendering individual water rations safe for consumption through chemical treatment, particularly with substances giving off chlorine (see summary by Kolle & Prigge, 1928, page 101), though of some value for armies in the field, are of little, if any, importance for protection during cholera epidemics. In regard to the recommendation of some workers to use acid drinks during such outbreaks, one ought to agree with the postulation of Strong (1944) that

"as a matter of fact the best prophylactic is the normal gastric juice, and there is a possibility that the use of such acid drinks might upset the digestion and defeat the object desired."[2]

As alluded to by Liebermeister, one point regarding which even people trying to beware of cholera infection are apt to err is that, while insisting

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[1] Santobquido (1913) referred in this connexion to an epidemic in an Italian asylum for the insane which in his opinion was terminated by keeping the patients confined to bed with their hands tied and untying and disinfecting the latter immediately before the meals.

Cartron (1915), feeling convinced that cleansing of the hands was essential for the control of cholera outbreaks, established *postes de savonnage* (washing stations), consisting of a supply of soap and of potassium permanganate solution in empty petrol tins fitted with a bamboo spigot, outside the houses of cholera patients or suspects, and also in military barracks, prisons and prison hospitals. The implementation of this method appeared to lessen the incidence of the disease.

[2] This objection probably also holds true as far as the prophylactic administration of aromatic sulfuric acid, recommended by some cholera workers, is concerned. Moreover Tomb (1926), experimenting with this drug, though claiming that it had considerable virtue for the treatment of the disease, found it practically valueless as a prophylactic.

be protected as well as possible against flies and other insects. It is best altogether to avoid the consumption of fresh fruits and in general of all uncooked dishes. Otherwise it is advisable to continue with the former mode of life, as far as it is adequate, but to avoid more carefully than before all that might cause digestive disturbances and particularly diarrhoea That the abundant consumption of alcoholic drinks, particularly of red wine, protects against cholera, is a superstition moderate amounts are innocuous for those used to them, an immoderate consumption is dangerous. Generally speaking one must beware of excesses of all kinds, also of catching colds and other unfavourable influences. Above all it is important to consider each diarrhoea becoming manifest in cholera times as a dangerous affection needing most careful treatment." [Trans.]

FIG 22. CARICATURE DEPICTING DIVERSE MEASURES FOR CHOLERA PREVENTION IN 1831 EPIDEMIC IN BERLIN

From the Kaiserin-Friedrich-Hauses collection, Berlin, by courtesy of Ciba Aktiengesellschaft Basle.

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As alluded to by Liebermeister one point regarding which even people trying to beware of cholera infection are apt to err is that, while insisting

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[1] *Samolqueldo* (1913) referred in this connexion to an epidemic in an Italian asylum for the insane which in his opinion was terminated by keeping the patients confined to bed with their hands tied and untying and disinfecting the latter immediately before the meals.

Cartron (1915), feeling convinced that cleansing of the hands wa essential for the control of cholera outbreaks, established *postes de savonnage* (washing stations), consisting of a supply of soap and of potassium permanganate solution in empty petrol tins fitted with a bamboo spigot, outside the houses of cholera patients or suspects, and also at military barracks, prisons and prison hospitals. The implementation of this method appeared to lessen the incidence of the disease.

[2] This objection probably also holds true as far as the prophylactic administration of aromatic sulfuric acid, recommended by some cholera workers, is concerned. Moreover Tomb (1926), experimenting with this drug, though claiming that it had considerable virtue for the treatment of the disease found it practically valueless as a prophylactic

upon the impeccability of their drinking water they pay little or even no attention to the equally important need of using boiled water exclusively for other household purposes, particularly for cleaning the eating and drinking utensils. Another often neglected potential source of danger is ice boxes, not only because they may be stocked with ice manufactured from unsafe water, but also because they may be used for storing cholera infected foods, particularly fish, which in their turn may be responsible for the contamination of articles such as butter (Strong, 1944). Henderson & Seneca (1951) were certainly right when stating that besides the ice itself "all contents of an ice refrigerator must be suspected"

While, as postulated by Liebermeister and other authors it is necessary carefully to attend to any diarrhoea attack observed during cholera out breaks, on the other hand every possible effort ought to be made on the part of the anti-epidemic staff and the medical profession in general to ensure that the people do not, instead of quietly and trustfully taking the reasonable precautions against the infection recommended above live in constant fear of the disease and, therefore, anxiously watch for any symptom that might herald its approach To allay such panicky fears on the part of the population is all the more important because their presence may lead to gastro-intestinal disturbances of a nervous nature

Public Health Education and Propaganda

Though imparting to the people a knowledge of the methods of personal prophylaxis just discussed forms a most important part of the public health propaganda campaigns to be conducted during cholera outbreaks, it is indispensable to enlist the co-operation of the public in other respects as well. Accordingly as Pollitzer (1948) briefly put it, it ought to be the aim of such campaigns to urge the people

1 Quickly to report instances suggestive of cholera and promptly to bring the patients for treatment.

2. To get vaccinated against cholera.

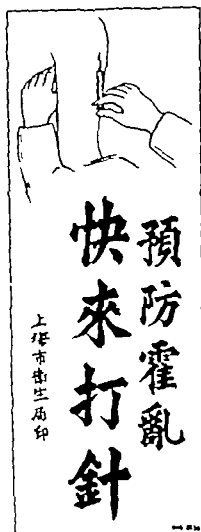
3 Strictly to avoid cold foods and cold drinks, partaking instead only of freshly prepared cooked food and boiled water or tea.

4 To use boiled hot water for cleaning cooking, eating and drinking utensils.

5 To keep the houses in a sanitary condition and to abate in particular the fly nuisance. Food and drinking water supplies as well as the eating and drinking utensils ought to be kept in fly-proof containers or under screens.

It is obvious that the results of propaganda work undertaken at the time of cholera outbreaks are bound to be optimal if that work represents merely an intensification and special adaptation of permanently conducted general public health education and propaganda campaigns, the aim of which is to impart to the people including the school youth a knowledge of the principles of hygiene

FIG 23. POSTER USED IN SHANGHAI IN THE 1930s URGING THE POPULATION TO BE INOCULATED AGAINST CHOLERA



Another point of vital importance for the success of the propaganda campaigns is that they must be carefully adapted to the level of the population groups which it is most essential to reach As far as anti cholera propaganda campaigns in particular are concerned one should not lose sight of the fact that generally speaking, the disease is apt to be most rampant among the less privileged classes of the population that is among people one may hope to reach with the aid of illustrated posters and handbills rather than with pamphlets and leaflets (Fig 23) Good advantage may be taken also of illustrating the methods of cholera prophylaxis with the aid of magic lantern or better still cinema shows which, being increasingly used for the purposes of general public health education and propaganda, are nowadays often available

While unillustrated lectures are of questionable value except for instructing selected groups for instance, officials or teachers in the affected areas, it is difficult to overrate the importance of imparting information on the methods of cholera prophylaxis through public health talks given to individual families or other small groups of the population As has been mentioned before such public health talks may be delivered during the off seasons by staff members visiting the houses in order to promote sanitary improvements or to give vaccinations During epidemics also every possible advantage should be taken of visits to the houses to tell the inmates in simple language what precautions they could take against the infection Moreover as has been recommended by some workers—by Mendelson & Tait (1921) for instance—special inspectors may be employed for house-to-house visits with the purpose of instructing the families concerned in the methods of cholera prophylaxis.

Mass Prophylaxis

Before dealing with the problems of vaccination by far the most important method of mass prophylaxis against cholera, it is proposed for the

convenience of record briefly to deal with three other methods which have been tried to ward off attacks of the disease—namely (1) administration of essential oil mixtures (2) recent attempts at chemoprophylaxis and (3) prophylactic use of bacteriophage

Administration of essential oils

As noted in Chapter 9 Tomb (1926) claimed to have obtained most satisfactory results when prophylactically administering the essential oils mixture recommended by him for early cholera treatment to numerous contacts of patients suffering from the disease Since however it is uncertain to what extent the clinical diagnosis of cholera as confirmed in the latter by laboratory examination and, more important still since the infection often fails to spread among contacts who did not receive any prophylactics, it is not possible to give much credence to Tomb s claims. Certainly the method of cholera prophylaxis recommended by him has not attracted any further attention

Chemoprophylaxis

A study of the prophylactic action of sulfadiazine in cholera was undertaken during the 1946 cholera epidemics in China by Peterson (1946) on a group of 2205 persons receiving 1 g of the drug per day for at least two days, whenever possible for five days, whereas a fully comparable control group of 2563 persons was given tablets containing an inert substance on the same schedule As shown by observations extending over six days, only two individuals of the treated group each of whom had received but one dose of the drug before the onset of symptoms, developed typical clinical signs of cholera whereas 11 attacks were noted in the controls Moreover unspecific gastro-intestinal upsets occurred in only 12 of the persons dosed with sulfadiazine, but among 41 of the controls.

Peterson expressed the hope that further studies would be made to establish more fully the prophylactic value of sulfadiazine or of other chemotherapeutic or antibiotic agents against cholera. In his opinion, however " no such agent, however successful it might be prophylactically will at present substitute for sanitation and vaccination."

Commenting on Peterson s work, Henderson & Seneca (1951) considered it desirable in cholera prophylaxis to use a less toxic sulfonamide than sulfadiazine particularly phthalyl sulfacetamide, which they claimed,

"is applicable to routine administration on a large scale without intermediate supervision, since no detectable blood concentration is set up and there is no conceivable hazard of crystalluria."

Hence, in the opinion of the two workers

"Controlled groups such as military personnel or hospital staffs, exposed to cholera in a region, can be afforded continuous protection from the first day by a standardized prophylactic dose such as 1 Gm daily"

However, they considered it problematical whether this method would find wide application in India.

Bacteriophage administration

In order to appreciate the importance ascribed by d Hérelle and some other workers to bacteriophages in the epidemiology and prophylaxis of cholera, attention may be drawn to a general postulation made by d Hérelle, Malone & Lahiri (1928) to the effect

"that in infectious diseases of the intestinal tract the onset and the course of the morbid processes are intimately associated with the behaviour of the pathogenic bacterium and of the intestinal bacteriophage towards one another and that recovery is not caused, as hitherto accepted, by a phenomenon of immunity but indeed by the action of a bacteriophage, whose virulence becomes exalted in the intestine of the patient and effects the destruction of the pathogenic germs"

In accordance with these contentions, d Hérelle & Malone (1927) maintained that bacteriophages virulent for *V cholerae* were absent in localities free from infection with this organism. If the disease appeared at first many patients, infected through direct contact or by means of water or flies, succumbed, because in them the intestinal bacteriophage remained inert towards the cholera vibrios However d Hérelle & Malone continued, there were others,

"in whom there is a rapid exaltation of the virulence of the normal intestinal bacteriophage towards the cholera vibrio and these individuals enter into convalescence. Virulent bacteriophages from these convalescents are passed with the stools, and are spread in exactly the same manner and by the same agents as are the pathogenic vibrios. In a word, at the beginning there are disseminated into the environment the cholera vibrios, and this is the period of the propagation of the disease. Then from the first convalescent there are disseminated the bacteriophages. As more and more patients recover the bacteriophages become more and more disseminated and the epidemic declines, finally to cease when contamination by the bacteriophage becomes general."

Fascinating as this hypothesis is, unfortunately many facts have become known which are not in agreement with it. Were the choleraphages as potent as has been claimed by d Hérelle and some other workers one would expect that they would invariably prove efficacious for the treatment of cholera Actually as has been discussed in Chapter 9 not only good but also disappointing results with this therapeutic method have been recorded, particularly by Taylor and colleagues (1930) who finding no phages active against the patient s own vibrios in the stools of recovering

cholera sufferers, came to the conclusion that bacteriophage was not an essential agent of recovery As will be shown now the prophylactic use of choleraphages has also not yielded uniformly good results and most of the modern workers are sceptical regarding the value of this method or even altogether deny its usefulness.

In their early reports on cholera prophylaxis with bacteriophages, d Hérelle Malone & Lahiri (1928) maintained

"that in villages where patients are treated by cultures of bacteriophages of exalted virulence an experiment in prophylaxis is instituted at the same time, for the exalted bacteriophages multiply in the intestines of convalescents are passed out with the stools and are disseminated into the environment as we have been able to verify on many occasions."

It was nevertheless evident

"that in these cases the diffusion of the exalted bacteriophages takes place more slowly than if the cultures are directly poured into the wells supplying drinking water and, furthermore, these bacteriophages are spread only in those regions of the villages where cases have been treated."

D Hérelle and his colleagues (1928 1930) were able to resort to the treatment of wells with choleraphages in three instances only Hence as they stated in 1930

"The number of villages in which we have been able to apply the method of collective prophylaxis by bacteriophage is certainly not large enough for any definite conclusions regarding its absolute efficacy to be drawn nevertheless it appears that these experiments tend to demonstrate it."

In addition to the method described above d Hérelle and co-workers also made an attempt at individual bacteriophage prophylaxis trying to induce the guests attending a marriage feast to drink a glass of water con taining 2 ml of choleraphage Among the 77 persons who agreed, only two developed mild and rapidly terminating cholera attacks, whereas among the 34 who refused the disease became manifest three times two of the sufferers succumbed, while the third, who received bacteriophage treat ment, survived. However suggestive though these observations seem at first glance they cannot be considered convincing in view of the usually quite irregular appearance of cholera among any given group of the population

A further and most favourable report on the results of bacteriophage treatment of wells in Puri Orissa, was rendered by Asheshov and co-workers (1930) who finding the cholera incidence to be ten times less in the area where 50-ml amounts of bacteriophage had twice been poured in than in the control areas (where well chlorination was practised) were led to think that bacteriophage prophylaxis was bound to prove a powerful weapon in controlling the spread of the disease in India.

The enthusiasm of Asheshov and his colleagues was not shared by Mackie, who in the course of a discussion reported in the *British Medical*

Journal (1933) stressed that a drop in the incidence of cholera as spectacular as that in the experimental area of Puri had been recorded in another area of Bihar and Orissa where no bacteriophage had been used

Nevertheless, as can be gathered from a report by Duggal (1949) bacteriophage administration remained for over a decade a most popular method in Bihar even though in the opinion of this observer

Probably the good results attributed to it were more due to the behaviour of epidemic periodicity rather than inherent useful properties of the Choleraphage."

Still Duggal continued, the method

"caught the fancy of both the Doctors and laymen so much that the orthodox anti-Cholera measures of disinfecting water supplies and anti-Cholera inoculations were at the stage of being totally substituted when the curve of the Cholera incidence started rising again in 1937 and reached its culmination in big epidemics of 1944 resulting in one and a half lacs [i.e. 150 000] deaths. During this period of rising curve Choleraphage was used practically to the exclusion of disinfection of water supplies, as such procedure was considered inimical to the efficacy of phage."

The sad experiences of 1944 gradually lessened the popularity of bacteriophage prophylaxis so that, as stated by Duggal now " the medical practitioners do not look upon it as a good anti-cholera measure " In Duggal s own opinion the prophylactic as well as the curative value of bacteriophage when used in the field was " a very doubtful entity "

As stated by Morison, Rice and Choudhury (1934) in continuation of work described by Morison in 1932 they had obtained most encouraging results in the prevention of cholera in certain villages of Assam through the distribution of cholera-dysentery bacteriophage by the inhabitants themselves to all patients suffering from diarrhoea, dysentery and suspected cholera. Summarizing the details of this work, Morison and colleagues recorded that the experimental area originally chosen, Nowgong,

" had a triennial death rate from cholera of 122.0 per 10 000 from 1906 to 1919 Between 1919 and 1929 the triennial death rate from cholera was 39.2 per 10 000 but epidemics still occurred with great regularity Since 1929 epidemic cholera was altogether absent, the total deaths from cholera during the three years were 53 47 and 27 respectively and the triennial death rate was 2.23 per 10,000 The first fall in the death rate in 1920 followed the great epidemic of 1919 compulsory vaccination of tea-garden coolies entering Assam and the use of vaccine in villages when cholera broke out The absence of epidemic cholera after 1929 followed the distribution of bacteriophage to the villages along the Kalang river where cholera was prone to occur and the withdrawal of vaccination, at first partial and later complete. Such absence of cholera during the last three and a half years did not occur in the arbitrarily selected control area Habiganj in the Surma Valley nor in the more appropriate control districts adjoining Nowgong with which, previous to 1929 there was a high correlation in the deaths from cholera."

In a further report on the work described above Morison (1935) recorded that Nowgong had remained free from epidemic cholera. In the control area of Habiganj outbreaks continued to appear each spring and autumn, until it was decided in July 1932 to start with phage distribution there as

well. As described by Morison, the results obtained in Habiganj were as satisfactory as those in Nowgong. He stressed in this connexion that

" data from 736 houses with a total population of 4 755 show that, in groups in all other respects alike, the reduction in the subsequent cases when the first cases in these houses received phage was 69 per cent "

and expressed the opinion that " it is to this reduction in infectivity that we must ascribe the results in Nowgong and Habiganj " [1]

While admitting that phage " is not the remedy for cholera in a city like Calcutta, nor is it a remedy where the source of infection can be traced and dealt with " Morison came to the conclusion that

" the widespread epidemic of cholera in various provinces of India compared with its control, where this has been attempted with phage in Assam, shows that it is a weapon we cannot yet afford to discard."

It is melancholy to note that further reports from Assam failed to confirm the favourable opinion held by Morison in regard to this method. Thus, as stated in a review of the 1935 report of the Shillong Institute in the *Tropical Diseases Bulletin* (1937)

" There is discernible an altogether hesitant note about the value of bacteriophage control in this report. The control areas of Nowgong and Habiganj do not seem to be furnishing unequivocal results, possibly because During an epidemic bacteriophage is being widely used along with preventive inoculation and other measures In these circumstances it is difficult to assess the value of bacteriophage as the sole measure in the prevention and control of cholera in the experimental areas If however the bacteriophage control has been effective in Nowgong it is reasonable to assume it should be effective in Habiganj also A study of the epidemic in the Surma Valley does not show conclusively that it had been so "

An even more pessimistic note was sounded in the review of the 1936 report of the Shillong Institute in the *Tropical Diseases Bulletin* (1938) wherein it was said that

" Comments and conclusions on the bacteriophage trial in Assam are depressing reading. The Director of the Institute states Had the use of bacteriophage been strictly confined to the experimental area we should, I believe, by now have reached a conclusive result. As it is, it appears unlikely that we shall ever do so by an experiment on these lines in Assam. "

As quoted by Pandit (1951) in view of this evidence the Cholera Advisory Committee of the Indian Research Fund Association reiterating in 1944 a recommendation made in 1934 expressed the opinion that bacteriophage prophylaxis should not replace the orthodox methods of cholera control, such as vaccination.

[1] As shown by the findings of Pandit & Rice (1936), this statement cannot be considered generally valid. It is also noteworthy that according to observations made by Pandit et al. (1936) a reduction of the infectivity was noticeable not only in the group of patients receiving early phage treatment, but also in groups treated with other remedies, particularly with essential oils. To explain these results, Pandit and his colleagues referred to the possibility that the families of cholera patients who resorted to any form of treatment presumably took precautions against a spread of the infection.

Attempts to make prophylactic use of choleraphages were also made by Raja (1934) in a district of the then Madras Presidency and by Boulnois (1936) at Chandernagore Bengal The last mentioned worker who as noted in Chapter 9 had obtained some satisfactory results with early bacteriophage administration to cholera patients also noted a marked reduction of the attack rate among contacts of sufferers to whom the phage had been given prophylactically Raja (1934) besides obtaining no success with bacteriophage tratment found that phaging of wells used in combination with oral phase administration to the population at large failed to reduce the cholera attack rate and was of doubtful value in reducing the mortality rate

The invariably more or less unfavourable references made by modern compilers to the bacteriophage prophylaxis of cholera may be summarized as follows

Author	*Statement*
Strong (1944)	Maintained that "the value of the use of bacteriophage in destroying the cholera spirillum in wells is still speculative and should not be relied upon"
Siler (1944)	Stated that the choleraphage studies made thus far "through promising in the nature of the results obtained, have not yet reached a stage that will permit drawing any definite conclusions as to their value either in curing or preventing cholera"
Napier (1946)	Summarized that "In Bihar and Assam, extensive trials were carried out in which bacteriophage was distributed for the treatment of their water supplies to a large number of villages in the areas where cholera occurs frequently The results appeared satisfactory but would not survive statistical crictitsm. In later years, the trials were repeated with disappointing results."
Rogers (1952)	Expressed the opinion that "The distribution of polyvalent bacteriophage cultures in the water supplies during outbreaks have yielded variable results. In Assam material benefits were claimed for their method of their use, but later inquiries have thrown doubts on the results owing to the absence of a strictly controlled basis for the tests. Trials in Bihar where the incidence of cholera is more uniform, failed to yield evidence of the value of the addition of bacteriophage to the water supply so it must be concluded that further carefully controlled trials during cholera epidemics are required to decide the value if any of bacteriophages in either the treatment or the prevention of cholera"

Notwithstanding the advice of some of the above-quoted authors, in view of the unfavourable evidence already available one must wonder whether there is really a need for further studies on the prophylactic administration of choleraphages.

Vaccination

Though as will be gathered from Chapter 4 various methods have been devised to induce an active immunity against cholera infection in

man only one of these procedures—namely parenteral administration of vaccines killed by heat or by chemical means—has been permanently adopted and therefore deserves further consideration at the present juncture It is proposed in this connexion first to deal with what one may call the practical aspects of cholera vaccination and then to turn attention to the much debated question of the value of this prophylactic method.

Practical aspects of cholera vaccination

Dosage As has been discussed in the fourth chapter in order to confer a solid immunity to cholera it is essential to use a vaccine with a sufficiently high titre (preferably one with a vibrio content of 8000 million per ml, as adopted in India) and most desirable to administer this in two doses at an interval of about 7 10 days. The adult doses to be used under these optimal conditions are 0 5 ml for the first and 1 ml for the second injection If however as is distressingly often the case, a one-dose system of cholera vaccination has to be implemented, standard vaccines with a vibrio content of 6000-8000 million per ml ought to be injected in 1 ml doses, or correspondingly larger amounts of less concentrated vaccines must be administered.

General agreement exists that in case of necessity initial cholera vaccination must be followed at yearly or whenever indicated even at half yearly intervals by the administration of booster doses consisting of 0 5 ml or as the present writer for one thinks preferable, of 1 ml doses of a potent vaccine.

Recommendations made regarding cholera vaccination of young children vary considerably some authors maintaining that it ought to be used even in the case of infants of less than a year old (from 3 months upwards according to Cardamatis, 1914) while Roy (1919a) sets the age limit as high as four years. Rogers (1921) considered vaccination of children under two years to be contra-indicated, but it is deserving of attention that other authorities—for instance, recently Taylor (1951)—are inclined to exclude only infants less than one year old.

While the necessity of administering reduced doses of cholera vaccines to children is generally realized, it is difficult to compare the specific recommendations made in this respect by the different writers, because they worked with vaccines of different titre Savas (1914), who evidently used a potent cholera vaccine, containing 4 mg of bacterial bodies per ml, tabulated the dosages for the successive age groups as follows

Age-group (years)	*Dosage for initial vaccination (ml)*
Less than 1	0 1
1 3	0.2
3-5	0.3
5-10	0 4
Over 10	0.5 (= adult dose)

Double these doses were used for second vaccinations, administered one week after the first injection.

Taking the recommended adult dose as a standard of comparison proportionate amounts of other potent vaccines may be utilized for cholera immunization of children in different age-groups However in view of the fact that they tolerate this method of immunization well, there is no need for anxiously restricting the vaccine amounts to be administered to children

Technique of vaccination While most workers are in favour of administering the cholera vaccine subcutaneously considerable variance of opinion exists regarding the most suitable site to be chosen for this purpose Some are in favour of giving the injections into the upper part of the breast, but other parts of the trunk have also been considered, Serkowsky (1906) for instance, advising that in the case of stout persons the vaccine should be injected into the back. However both for the sake of expediency and in order not to add to the prejudices of the people against cholera immunization, most workers prefer to give the injections into the arm Cardamatis (1914), while maintaining, in agreement with many other observers, that the outer posterior aspect of the upper arm was most suitable for vaccination considered it permissible in the case of Mohammedan women for the injections to be given in the forearm However as will be further mentioned below difficulties arising in connexion with the vaccination of females are best solved by having female staff members available during the vaccination campaigns.

Since even quite modern instances could be quoted in which cholera vaccinations were administered with utter disregard of antiseptic precautions, it is necessary to insist upon the absolute need for proper sterilization of the skin site chosen for injection and the use of a freshly boiled or otherwise heat sterilized needle for each individual to be vaccinated The accidents which are well nigh inevitable if these elementary precautions are disregarded not only cause much individual suffering, but are apt to prejudice the people in general against the method of vaccine prophylaxis

Contra-indications As generally agreed the administration of cholera vaccines is contra indicated in the case of individuals suffering from (*a*) acute feverish diseases, and (*b*) serious chronic affections, particularly those of the kidneys or the heart. During cholera epidemics in particular one must also avoid vaccination of persons suffering from gastro-intestinal disturbances, especially diarrhoea. Such individuals ought to be kept under observation in order either rapidly to isolate and treat them should they be found to suffer from cholera, or to vaccinate them as soon as normal function of their gastro-intestinal tract has become restored.

Reactions Though the administration particularly the initial injection of a potent cholera vaccine is bound to lead to some local reaction and this may be followed by a rise of the body temperature or other signs of

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5-10	0 4
Over 10	0.5 (= adult dose)

Double these doses were used for second vaccinations, administered one week after the first injection.

For, as the two authors explained

"The supernatant fluid rejected at this stage carries away most of the nutrient materials and soluble toxins. If on account of carelessness this step is not properly carried out an excess of the latter may get into the vaccine and give rise to unpleasant reactions."

It is interesting to note that, in addition to the early reactions described above, secondary or late reactions to cholera vaccination have occasionally been described. Thus Tewari (1936) claimed to have observed in 8%-10% of the 4000 individuals vaccinated by him against cholera secondary reactions which appeared on the 12th, 13th or 14th day after immunization and consisted of pyrexia varying from 100° to 102°F (37.8°-38.9°C) as well as of redness, pain and tenderness to pressure at the site of injection. Tewari felt convinced that these reactions, which were milder than those immediately following vaccination, lasting only for 28-36 hours, were due neither to contamination of the vaccine (obtained from a first-class laboratory) nor to a faulty technique of injection.

Referring evidently to a different phenomenon, Taylor (1951) stated that in a small proportion of cholera vaccinated individuals " a late reaction may occur about the eighth day after injection with puffy swelling at the site of inoculation which subsides in 36 hours without treatment."

Turning attention to unusual or particularly severe reactions occasionally met with after cholera vaccination, record has to be made first of observations of Dedekind (1915), who described 18 instances of severe cellulitis, requiring incision and drainage, in a regiment of soldiers vaccinated with unsterilized syringes and needles and without any skin disinfection.

Wenderoth (1943) referred to three instances in which cholera vaccination was followed (in the two well-observed patients within a few days) by the appearance of marked skin erythema associated with eosinophilia and in one of the patients also with skin haemorrhages and a serious general condition.[1] Since in two of these instances intracutaneous injection with small doses of diluted cholera vaccine led to a marked local reaction, Wenderoth ascribed these incidents, recovery from which required about two weeks, to an individual primary hypersensitivity to the *V. cholerae* protein.

The occurrence of anaphylactic shock in three out of about 25 individuals who had been vaccinated against cholera in 1913 and 1914 and were revaccinated in October 1915 was claimed by Parhon & Bazgan (1916). Commenting upon these observations, Papamarku (1917) warned against unnecessarily frequent cholera vaccination. He admitted, however, that in localities with two cholera seasons per year it might become necessary to give more than one yearly booster dose. One might add that in view of the extreme rarity of such anaphylactic reactions or of signs of hyper-

[1] The observations made in this patient are quoted in the review of Wenderoth's article in *Trop. Dis. Bull.* 1944, 41, 130.

a general response to the immunization, one must fully agree with Taylor (1951) that, as a rule "the reaction, either local or general, produced by the vaccine is negligible and inoculated persons can carry on with their work."

Early reference to this fortunate absence of "unpleasant or dangerous" symptoms after cholera vaccination was made by Murata (1904) according to whose pioneer observations the reactions seen after parenteral administration of a heat killed and phenolized vaccine were as follows

"(1) The body temperature showed mostly 38.0°C or very rarely 39.0°C, an increase which did not last longer than 24 hours. Sensation of chills was very rare. (2) 5-6 hours after vaccination spontaneous painfulness or sensitivity to pressure became noticeable at the site of injection. The local swelling and redness were mostly insignificant. If present, they disappeared at the latest after 3 days. More rarely I observed urticaria also. (3) After vaccination the amount of urine became increased (in about 20% of all cases) but became normal once more after 12-16 hours.[1] In a few instances (about 10% of all cases) 1-2 diarrhoeic stools were voided on the day following vaccination. (4) In women one could observe nausea and vomiting. (5) Other usual complaints included general lassitude, malaise and headache." [Trans.]

In agreement with these observations, Savas (1914) reporting on large-scale vaccination campaigns in Greece at the time of the 1913 cholera outbreak, stated that

"The rare complications which sometimes accompanied the vaccination were a slight local reaction, a fever which but exceptionally became higher than 39°, lassitude and, in some cases, vomiting and diarrhoea." [Trans.]

Comparing different cholera vaccines, Babes (1914) found that some of them produced more violent reactions, consisting of higher fever, marked nausea, vomiting, diarrhoea, exhaustion and longer persistence of the symptoms. He maintained that differences in the "irritating action" of the various strains used for the manufacture of the vaccines were responsible for the marked reactions produced by some of the latter and, as noted in Chapter 4, therefore stressed the necessity of separately testing each culture it was proposed to use for the production of polyvalent cholera vaccines.

In contrast to these postulations, Maitra & Ahuja (1931) found that, given the absence of contamination of the brews and a proper technique of injection, the untoward reactions following cholera vaccination were due to the presence of foreign proteins and soluble products of bacterial metabolism in the vaccine fluids. They insisted, therefore, that the washings from the agar cultures which had been sown with *V. cholerae* for the purpose of vaccine manufacture be

"allowed to stand for 48 hours to enable the suspended bacteria to be deposited. The supernatant fluid is then decanted off and the bacterial deposit retained for preparing the vaccine."

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[1] Balteanu & Lupu (1914) maintained that, though polyuria was more often observed, anuria was occasionally present after cholera vaccination. Albuminuria or casts appeared to be absent in the urine of vaccinated persons.

to make ample use of this prophylactic method At the same time however it was maintained there that

"both in order to benefit as many persons as possible and to avoid alarming the people by the occurrence of the disease in recently vaccinated individuals every possible effort should be made to administer the vaccinations before onset of the cholera seasons"

At first glance it would seem that in order to benefit as many people as possible it would be desirable to make cholera vaccination compulsory whenever outbreaks are present or imminent Actual experiences have shown however that one should not be rash in insisting upon enforced wholesale vaccination both because (*a*) those averse to this method will resort to every possible subterfuge to avoid being vaccinated thus thwarting the desired object and because (*b*) the use of police methods to enforce a public health measure is apt to militate against the friendly relationship between the anti-epidemic staff and the people which for many reasons it is most essential to establish Hence though situations might arise in which the need for enforced wholesale cholera vaccination is so overwhelming as to override any objection to this method it is generally wiser to persuade rather than to compel the people to get vaccinated All suitable means of propaganda must be used, therefore to make the method popular but, as far as the present writer can judge from his own experience the best means to provide for large scale immunization is the adoption of a system of house-to-house cholera vaccination As has been noted above it may be essential to include female nurses in the squads used for this purpose because in some countries the people resent having the female members of their family treated by male staff members or even refuse to have this done As far as the experiences in China went it was found best to utilize female nurses for vaccination of the males as well because these nurses were much more adept in obtaining the consent of the people than the male staff members who becoming impatient far more easily were apt to enter into undesirable arguments

In rural areas, where it may be difficult, if not impossible to use a system of house to-house vaccination advantage may be taken of markets or other assemblies of the people to offer opportunities for immunization against cholera

However much one may be inclined to object to the use of compulsion in general vaccination campaigns, one must agree that some system of enforced vaccination against cholera has to be adopted when one is called upon to deal with groups of people like seasonal labourers or pilgrims [1]

Compulsory immunization of seasonal labourers en route from various cholera affected areas in India to Assam seems to have been adopted first

[1] Compulsory vaccination against cholera has also been recommended for other groups of people, for instance, according to Takano and co-authors (1926), for fish merchants, fishermen, sailors and boatmen in Japan. The use of the method in international quarantine practice will be discussed in the concluding section of this chapter

sensitivity caused by cholera vaccination, one should certainly not hesitate to take reasonable advantage of this prophylactic method whenever its implementation is indicated

Interesting observations on the occurrence of a condition simulating appendicitis in 38 out of a group of 1100 men vaccinated against cholera have been recorded by Woodward (1946) these rather alarming symptoms following the initial inoculation in 20 instances and appearing after the second vaccine injection 18 times Details of Woodward s observations were as follows

"The symptoms in order of prominence and frequency consisted of abdominal pain in the right lower quadrant of the abdomen or in the region of the umbilicus (a few also had epigastric or left lower quadrant pain) diarrhoea, constipation (or both, alternating) vomiting, headache, and several had symptoms of a mild upper respiratory infection. The temperature was elevated from ½ to 1½ degrees in practically all cases."

Objectively one found

"abdominal tenderness, at McBurney s point or slightly medial to it, and also in the epigastrium or left lower quadrant in several cases. Muscular rigidity was infrequent and was only slight when present. Right-sided rectal tenderness was a constant finding, with tenderness also on the left in the cases showing abdominal tenderness in the left lower quadrant."

A leucocytosis, averaging approximately 10 000 was invariably present, but differential counts revealed no unusual findings Analysis of the urine showed nothing abnormal and, except for the presence of mucus nothing peculiar was found in the diarrhoeic stools Appendectomy was performed in three instances with the result that

"there was only a slight appendiceal inflammation in 2 of the cases, being rather a peri appendicitis and hardly greater than that present in the cecum and terminal ileum. The third case had a marked injection and edema of the appendix. All the cases revealed a marked mesenteric lymphadenitis."

In the other individuals showing the above syndrome (two of whom had had a previous appendectomy) the signs which had become manifest from two to five days after vaccination, spontaneously disappeared after an average of approximately a week

One must fully agree with the postulation of Woodward that the unusual incidents observed by him stood in causal connexion with the administration of cholera vaccine Quite possibly an inflammation of the mesenteric lymph nodes as was actually observed in three instances, was the primary cause of these reactions

Administrative arrangements In the conviction that cholera vaccination is not followed by a "negative phase" during which a temporarily increased susceptibility to the infection is present it was stressed in Chapter 4 that in the unforeseen calamity of a cholera outbreak one should not hesitate

the value of the method were not statistically significant An exception seemed to be formed however by observations recorded by Savas (1914) in regard to the sanitary corps of the Greek Army As Greenwood & Yule pointed out it seemed reasonable to think that the members of this corps

" did not occupy a more but a less favourable position than the combatants as regards the risk of infection the only particular in which they were distinguished from all or nearly all the combatants was, that practically every man had been twice vaccinated before the disease broke out."

That their fully adequate immunization with an agar grown and heat killed cholera vaccine indeed gave excellent protection to the members of the sanitary corps is shown by the following statistics representing Savas s data as summarized by Greenwood & Yule

	Not attacked	*Attacked by cholera*	*Total*
Sanitary corps	2 884	13	2 897
Combatants	112 613	2 192	114 805
Total	115 497	2 205	117 702

Commenting upon these statistics, Greenwood & Yule stated

" That anti-vaccinists will accept our conclusions respecting Savas s data is wildly improbable. We know no more about the Greek Sanitary Corps than Savas tells us. They may all have been vegetarians, or non-smokers, or red-headed and all or any of these things may render them less likely to contract cholera, but we do not see why objections which no sensible man would allow to influence him in the affairs of ordinary life should suddenly acquire scientific importance when the question is one of interpreting statistics. Our conclusions, then, respecting the Greek experience are that, although no inference can be drawn from a comparison of the attack-rates upon inoculated and uninoculated soldiers in the combatant units, yet the striking difference between the incidence upon the sanitary corps and that upon the rest of the army is evidence in favour of the efficacy of the process."

Cholera vaccination amply used by Savas and other workers during the Balkan wars was also widely practised in various of the armies participating in the First World War but to judge from the conflicting statements of Hetsch (1928) who strongly upheld the value of the method and of Harvey (1929) who was quite sceptical no unequivocally convincing statistical evidence for the efficacy of parenteral cholera immunization was furnished. However as aptly pointed out by Hetsch conclusions had to be based not only upon large scale statistics, but also upon observations in single families or in small easily supervisable population groups, which were repeatedly found to confirm the value of the method in a manner almost tantamount to that of laboratory experiments. The following records of this nature deserve special mention

Author	*Observations*
Murata (1904)	Reported that (*a*) out of 159 staff members of an office all but three were vaccinated according to Kolle s method. One of these three individuals contracted cholera to which he suc

in 1919 and, to judge from the following figures furnished by Young for that year it proved an outstanding success from the beginning

	Total cholera mortality in the recruiting areas in India	*Weekly number of emigrants*	*Cholera deaths en route from India to Assam*	
			number	*ratio per mille*
Prior to vaccination (January 22 March)	27 825	106 934	726	6.78
After introduction of vaccination (29 March-31 May)	65 970	89 609	166	1.8

It will be noted that, though the cholera mortality in the recruiting areas (Madras, Bengal, United Provinces Bihar and Orissa, and the Central Provinces) after the introduction of vaccination of the seasonal labourers was more than double that of the pre vaccination period, immunization of these individuals led to a marked drop of the cholera deaths en route

In agreement with these experiences White (1923) was informed that cholera vaccination had " proved its value in connection with the despatch of Annamite labour forces overseas "

The ample use which has been made of the method of compulsorily vaccinating pilgrims intending to attend fairs and festivals in India will be discussed in a later section of this chapter As will be shown, such campaigns have invariably been spectacularly successful, thus lending strong support to the evidence which will now be presented. Attention will also be drawn to equally satisfactory results obtained with vaccination of groups intending to make pilgrimages to Mecca.

Value of parenteral cholera immunization

Studying the most voluminous literature dealing with active immunization against cholera with the aid of the generally accepted method of using killed vaccines, one finds that the various authors treating this subject fall into three groups—namely those who come to the conclusion that this mode of vaccination is definitely useful for the prevention and control of cholera, those who express a directly opposite opinion, and finally a number of writers who while not denying the value of the method, feel doubtful as to its actual importance in anti-epidemic work Since it would be neither possible nor useful to enter at the present juncture into a detailed analysis of the copious writings of each of these schools the present author proposes instead succinctly to set forth the reasons why he feels convinced that parenteral cholera immunization with killed vaccines, though far from being a panacea, is of great value for the prevention and control of manifestations of the disease

Greenwood & Yule (1915) assailing the important early data on cholera vaccination with the heavy artillery of elaborate statistical methods came to the conclusion that in general the results, though slightly in favour of

Author	Observations
Wardener (1946)	Observed during a cholera outbreak among British and Dutch prisoners-of war in Siam an "almost complete immunity of the repeatedly inoculated Dutch although they were subjected to the same predisposing conditions and open to the same risk of infection"
Rogers (1952)	Mentioned that "in an outbreak of cholera in a large Bengal village at first only the Hindus submitted to inoculation and the disease ceased among them within a few days. The Muslim males then submitted to inoculation with a similar result but cholera cases continued among the unprotected Muslim females until they also were inoculated"

As has been discussed already in Chapter 4 large-scale and carefully checked statistics published by Russell (1928) again illustrated the efficacy of parenteral cholera vaccination which even if used according to a single dose system gave results fully comparable with those of oral adminis tration of bilivaccin Parenteral administration of two doses of vaccine was found to be markedly superior in value to that of ingestion of three doses of bilivaccin as far as the percentage mortality among those contract ing cholera infection in spite of immunization was concern d

A further report on a *large scale evaluation* of parenteral cholera vaccination was published by Adiseshan, Pandit & Venkatraman in 1947 supplemented by an elaborate statistical assessment of part of these findings by Chandra Sekar (1947) The main results which the first three workers obtained when administering a vaccine containing both the Inaba and Ogawa types of *V cholerae* with a titre of 8000 million organisms in single adult doses of 1 ml were as follows

(*a*) "There were 1 118 cases of cholera amongst 709 977 protected persons in the inoculated population and 34 336 cases of cholera in 2,119,568 uninoculated persons. The case incidence rates in these two groups of population were 1.57 and 16.20 respectively per 1,000 representing a ratio of 1 10 3"

(*b*) "Two or more outbreaks of cholera occurred in 627 out of 2,350 villages in the survey In these villages, the uninoculated population in the second and later outbreaks was 541,808 and the protected population was 281 484 all of whom had been inoculated during the first outbreak In the second and subsequent outbreaks 6,580 cases of cholera occurred in the uninoculated group and 241 in the protected group The incidence rates per 1,000 of the respective population groups were 12.14 and 0.86, that is, the incidence in the uninoculated was 14.2 times greater than in the protected group"

(*c*) "In South Arcot district which contributed over 50 per cent of the statistical material in the present inquiry there were 2,439 cases and 1 124 deaths in the inoculated group and 14 015 cases and 8,956 deaths in the uninoculated group The case fatality rates in the two groups are 46.08 and 63 90 per cent respectively the proportion being 1 1.39 [1] There is no significant difference in the fatality rates among the cases occurring in the successive days after inoculations."

[1] According to Chandra Sekar there was no significant difference in the case fatality rates among those attacked by cholera in the vaccinated and non-vaccinated population groups which he studied.

Author	*Observations*
Murata (1904) *(continued)*	cumbed, whereas all the vaccinated persons remained healthy (*b*) in another group of 100 employees only the single individual who had refused vaccination became cholera-infected and (*c*) the same held true of the wife of an official who, in contrast to her family refused to be immunized.
Nijland (1913)	Drew attention to a marked difference in the occurrence of cholera among the 8000 European inhabitants of Batavia, Java, who had been vaccinated, and the 2700 who had not been immunized. Only three cholera attacks were noted among the former and there was only one fatality in one of the three, who fell ill two days after vaccination. Among the non-vaccinated there were 32 instances of cholera with 15 deaths.
Savas (1914)	Noted that as soon as cholera vaccination had been completed in the Greek army the disease " disappeared as if by magic, and one found only some sporadic cases among soldiers who had been sent to fill gaps and who at first arrived without being vaccinated for it was only later that care was taken to send to the troops in the field only vaccinated soldiers. After vaccination of the latter had been terminated, cholera disappeared completely " [Trans.]
Cantacuzène (1920)	Referred to observations in a regiment, in which only the Jewish soldiers (numbering over 200) were immunized against cholera. While these men remained well, 450 of their unvaccinated comrades contracted the infection.
White (1923)	Was informed that police and Government servants in Indo-China who are protected by inoculation have enjoyed a striking immunity to cholera in the presence of infection, though their environment and mode of life are in all respects comparable to those of the general population among whom they live "
Mallik (1928)	As summarized in the Tropical Diseases Bulletin (1928) During the last six years the author has been using anticholera vaccine for the prevention of cholera among Europeans and certain classes of Indian employees of a jute mill in Calcutta. In all 420 inoculations were carried out during the six years, distributed among a total personnel and establishment of 280 Among these 280 there were 32 cases of cholera in six years, but in every instance the patient was a non-inoculated person."
Robertson & Pollitzer (1939) [1]	Recorded that Sometimes non-inoculated individuals amongst a group of inoculated persons were the only ones to become infected with cholera though all were exposed to the same risk. Thus at an ammunition factory forty guards were inoculated. One sentry was not able to come up for inoculation and he was the only one to become infected with cholera about 1 month later Factors such as food and water were identical for the whole group "

[1] For the convenience of the record this and the following later observations have been inserted in the tabulation.

Expressing this important statement in other words one should not hesiatte to make the best possible use of cholera vaccination whenever the situation warrants it but should at the same time never lose sight of the fact that this palliative method is not only incapable of eradicating cholera but is even less effective in checking the spread of the infection than the implementation of sanitary measures, particularly the provision of safe water supplies

Control of Pilgrimages [1]

Though, as Lal (1937) aptly stated when dealing with the problem of pilgrimages in India

"the importance of the fairs in the spread of infection, particularly of cholera, was recognized long ago it was only in recent years that earnest attempts were made to control them. People were used to *looking upon these happenings as inevitable and the* magnitude of the problem used to scare away the non-over-enthusiastic health officials."

Thus it came about that as Lal continued,

"*Up to the beginning of the present century the state responsibility in the control of the* infectious diseases was only partially recognized and it was left mainly to the local authorities to do what they could in the way of sanitary control of the fairs. Sanitary inspectors were put in charge of public health organizations at the fair grounds and their efforts usually ended in complete failure Apart from the insufficiency of funds and the lack of expert direction, the main reason for these failures was that no attempt was made to control the nodal points."

Evidently the pilgrim committees, which the Government of India alarmed by the role played by the pilgrimages in the spread of cholera appointed in 1912 in the provinces concerned (see Banerjea, 1951) recognized the need for action not only at the places where the fairs and festivals were held but also along the routes by which the pilgrims came and went For as Lal stated the reports of the committees, forwarded in 1916 by the Sanitary Commissioner to the Government of India, recommended that facilities for the isolation of pilgrims attacked by infectious diseases ought to be created at suitable stations of the main railway lines leading to the pilgrimage centres. At the same time the necessity of substantially improving the sanitary arrangements at the latter was stressed and to judge from the descriptions of authors like Dunn & Khan (1928) Lal (1937) and Yajnik & Prasad (1954) as well as from what the present writer had an opportunity of personally seeing in India, these arrangements gradually reached an admirable degree of perfection However Russell, in a lecture given in 1934 while also praising this work deplored the great difficulties and the considerable expenditure involved in making sanitary arrangements for the

[1] It is proposed to deal at the present juncture only with the control of pilgrimages in India, deferring discussion of the measures taken in regard to the Mecca pilgrimage to the section on international quarantine

(*d*) Judged by the incidence of the disease in the vaccinated persons, immunity against cholera began to manifest itself on the fourth day after vaccination and reached an effective level after the eighth day The immunity appeared to last for a minimum period of six months, but probably remained effective up to 12 months.

(*e*) " Herd immunity seems to play an important part in preventing multiple outbreaks in a locality during an epidemic. If during the first outbreak, 50 per cent or more of the population at risk is inoculated, the chances of subsequent outbreaks are greatly reduced."

The last mentioned observations fully supported the statement made in 1928 by Hetsch to the effect that

> " It is characteristic of the action of cholera vaccination that it by no means reliably protects all vaccinated, who afterwards become infected, but it does with safety inhibit the rise of epidemics if the threatened masses of the population have been well immunized [*durchgeimpft*]." [Trans.]

It is important to note that this " inhibitory " action of cholera vaccination is apt to become manifest even in the course of the outbreaks during which immunization has been started Some evidence to that effect was quoted by Rogers (1921) who mentioned *inter alia* (*a*) that in 1914 and 1915 cholera epidemics in Batavia decreased rapidly after 90% and 80% respectively of the population had been vaccinated, and less quickly where only 50% of the people were thus protected and that (*b*) according to Roy (1919b) wholesale administration of a potent cholera vaccine in an Indian village was followed by a rapid decline of an outbreak of such severity that " all ordinary sanitary measures in as far as they could be successfully applied absolutely failed not only to check but even to modify the epidemic "

Observations identical with those quoted above were made by Benjamin (1949) who stated that

> " In our experience we find that when no general preventive sanitary measures can be taken, if mass inoculation of about 70% of the population is done, the outbreak is controlled and subsides quickly "

Dealing in a general manner with the problem at present under review Burrows (1948) excellently defined the relative merits of sanitary measures and of vaccination in the fight against cholera by stating that

> " While the spread of cholera in epidemic form is readily preventable by the usual sanitary means, the disease becomes a menace when these break down. Such measures are not effective under the relatively primitive conditions prevailing in large parts of India and elsewhere in the Far East, nor during periods of social disturbance such as war in which large bodies of susceptible persons, either armed forces or displaced persons are living under highly unsanitary conditions. Under these circumstances, the prevention of cholera would seem to be largely a matter of effective prophylactic inoculation. For example in India at the present time the obvious solution to the spread of epidemic cholera is the development of adequate sanitary facilities, but prophylactic inoculation, if reasonably effective, may serve to bridge the gap between present primitive conditions and such social organization."

Inoculation of Central Provinces pilgrims has not solved the problem of the introduction of cholera by pilgrims."

A system of what Banerjea (1951) calls indirect compulsory vaccination against cholera by which all non immunized persons were prohibited from entering the locale of a pilgrim festival was first enforced at the 1936 Pandharpur fair Referring to this important event Benjamin (1949) stated that in that year cholera suddenly broke out in Bombay Province (now Bombay State) after the processions the so-called *palkies*[1] had started for Pandharpur several villages on the routes of the pilgrims be coming infected Since it was not always possible to divert the *palkies* it was decided to protect the pilgrims through cholera vaccination Results were so satisfactory[2] that it was decided to make vaccination compulsory for the Ashadi fairs which formed the goal of the processions. As Benjamin added

"This was not so for the Kartiki fair which is annually held in the off-season for cholera (November) but in view of the experience of the Kartiki fair of 1940 when cholera broke out at Pandharpur and was transmitted to several parts of the province, C.P [Central Provinces] and Hyderabad State, inoculation has been made compulsory for pilgrims visiting all fairs in Pandharpur Inoculation is also made compulsory for other fairs in the province whenever the area where the fair is held or from which the pilgrims come is infected."

Though as recorded by Banerjea (1951) and other authors the Indian Central Advisory Board of Health impressed by the success obtained in connexion with the Pandharpur pilgrimages recommended in 1940 to the provincial and State governments the introduction of an indirect form of compulsory cholera vaccination at selected festival centres, it was only in 1945 that the gigantic work of adopting this system for the pilgrimages in the United Provinces (afterwards Uttar Pradesh) was commenced. The results obtained then and during the following years including 1950 have been summarized by Gopal (1951) in the form of a table (see Table XXI) The observations embodied there leave no room for doubt that, in contrast to voluntary vaccination compulsory immunization of the pilgrims intend ing to visit the fairs was an eminently successful method. Indeed, one might say that the heroic efforts made in this direction, particularly on the occasion of the 1950 Kumbh fair at Hardwar won battles which, unlike those fought during wars saved numerous lives instead of sacrificing them.

To judge from apparently incomplete data furnished by Yajnik & Prasad (1954) compulsory vaccination against cholera was again used on the occasion of the 1954 Kumbh fair at Allahabad the number of inoculations

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[1] Reference to these processions has been made in the preceding chapter

[2] See the 1944 paper of Rogers, which contains a table comparing the incidence of cholera at the Pandharpur fairs and the spread of the infection from there by the pilgrims before and after the adoption of compulsory vaccination (periods from 1930 to 1935 and from 1936 to 1941). However evaluating these figures, it has to be kept in mind that, as described by Benjamin, fully adequate sanitary precautions, including the provision of safe water supplies, were taken during the *palkies*.

major fairs and festivals and admitted that besides these improvements the unusually low incidence of cholera in India during the previous two or three years might have been of importance for the absence of spread of the infection through the pilgrimages Indeed that measures of sanitation alone, however excellent, cannot control the emergency situations created by the pilgrimages seems to be proved by the outstandingly good results obtained with the compulsory vaccination of pilgrims which will now be discussed

A proposal to resort to mass inoculation of pilgrims seems to have been made first by Rogers, the pioneer in this field, in a paper published in 1926, wherein he claimed that

"Fortunately we now possess in inoculation against cholera a simple and effective method of protecting the pilgrims and other travellers against infection of themselves and, even more important, of bringing back the infection to their households and places of residence."

In a further article published in the following year (1927) Rogers stated more explicitly that

"the one great outstanding factor in the spread of cholera which can be controlled, is the movement of the twenty million pilgrims yearly in India, mostly through infected endemic areas, for we cannot influence the favouring effects of deficient rainfall, although we can watch it and be forewarned several months ahead of the danger of increased cholera in different areas following diminished autumn and winter rains, and we can also watch the daily and monthly absolute humidity in each province, so as to know when it rises to a point favouring the spread of the disease in each area, and consequently we can foresee when the journeys of the pilgrims traversing any given area at any given time of the year are likely to be especially dangerous, making it highly desirable to protect the pilgrims and other travellers through such localities."

As Rogers deplored in a paper published in 1944 his hopes that the pilgrims from the Punjab intending to attend the Kumbh fair at Hardwar in 1927 would be vaccinated against cholera were not realized, and as a result an epidemic took place with the highest provincial death-rate from cholera since the preceding Hardwar Kumbh fair in 1915 Similarly the authorities considering it impracticable to vaccinate the three million pilgrims expected at the 1930 Allahabad Kumbh fair disastrous outbreaks followed "with 147 000 deaths from cholera that year in Bihar nearly 60 000 in one month, together with 30 000 deaths in the neighbouring eastern divisions of the United Provinces"

According to Rogers (1944) the first serious attempt to vaccinate intending pilgrims against cholera was made in 1930 in the Central Provinces, where facilities for voluntary immunization were provided at hospitals, dispensaries and railway junctions. Though this campaign and those conducted during the following years seemed to have been attended by some success nevertheless, Rogers maintained,

"the provincial reports from 1931 to 1939 show importations of cholera into the Central Provinces every year It must, therefore, be concluded that the system of voluntary

with single 1 ml doses of the vaccine administered in the immediate vicinity of the fair area alone amounting to 223 350 As the two authors stated no epidemic of true cholera ensued either within or outside Uttar Pradesh However evaluating this fortunate outcome, it has to be noted that (a) the State had been free from cholera at the beginning of the fair and (b) an excellent programme of sanitary measures (including the provision of chlorinated piped water, adequate night soil disposal and extensive use of DDT) was implemented It was no doubt due to these measures that an outbreak of mild gastro-enteritis observed at the time of the fair showed no tendency to spread It is interesting to note that cholera like vibrios were isolated from 53 of the 145 stool samples collected from patients who suffered from such gastro-intestinal disturbances

Dealing with the problem of control of the pilgrimages in Orissa, Hajra (1949) reported that

" Sanitary arrangements at fairs and festivals, particularly at Puri, have been considerably improved during recent years In addition to the amounts spent by local bodies, Provincial Government have also come forward with grants to make more efficient sanitary arrangements during the festivals. Besides, ordinances are promulgated during certain years under the epidemic diseases Act of India making anti-cholera inoculation compulsory both for the residents as well as for the pilgrims during the Rath Jatra festivals. As a result of these arrangements the incidence of cholera at Puri during the festivals and its spread to other parts after the festivals have been greatly reduced. The arrangements made during the recent Rathajatra festivals were so efficient that the festivals passed off peacefully with only 11 attacks and 3 deaths from cholera during the entire festival period lasting for a month. No epidemic of cholera broke out in any part of the Province by the returning pilgrims even after the festivals."

Duggal (1949) reporting on the cholera problem in Bihar declared it impossible to use compulsory vaccination of the pilgrims " on account of lack of desire for this on the part of the people " He claimed, however that vaccination of as many pilgrims as possible, used in combination with adequate sanitary arrangements gave fairly satisfactory results, outbreaks of cholera traceable to the fairs having become infrequent. On the other hand an unexpected big religious gathering in 1946 in regard to which no precautions could be taken was followed by a major cholera outbreak in the surrounding districts

Commenting again on the excellent results of wholesale vaccination of the pilgrims a measure which he had so meritoriously recommended long ago Rogers (1957) expressed the belief that

" cholera incidence in India as a whole is gradually being brought under control by the general adoption of compulsory inoculations of the immense numbers of moving bands of pilgrims throughout India "

He admitted however that the unusual absence of any material failure of the monsoon rains during the last decade had favoured the remarkable decline of the disease

TABLE XXI. RESULTS OF VACCINATION TO CONTROL CHOLERA AT FAIRS IN UTTAR PRADESH

Name, place and date of fair	Approximate gathering	System of vaccination	Pilgrims vaccinated		Cholera incidence at fair		Effect on neighbouring districts
			number	%	attacks	deaths	
Ardh-Kumbh fair Hardwar, March April 1945	270 000 (on the main day)	Compulsory	318 630	98.00	—	—	No spread of the infection from the fair to the United Provinces or the Punjab
Ardh-Kumbh fair Allahabad, January February 1946	300 000	Voluntary	7 646	0.50	267	157	Widespread explosive epidemics caused by returning pilgrims in the eastern and central parts of the United Provinces
Sawan Jhula, Ayodhya, 7-10 August 1948	250 000	Compulsory	148 662	75.00	—	—	No effect in the province although the disease was being reported from all the neighbouring districts in the first week of August 1948.
Chaitra Ram Naumi fair, April 1949	100 000	Voluntary	40 019	5.00	103	40	Widespread explosive outbreaks caused through returning pilgrims in the neighbouring districts
Debi Patan fair March-April 1949	170 000	Compulsory	140 000	90.00	1 [b]	—	No spread of infection took place from this fair to any district
Sawan Jhula fair July-August 1949	180 000	Compulsory	94 048	63.00	—	—	No spread of the infection from the fair
Vrindaban Kumbh, January March 1950	150 000	Compulsory	43 417	32.27	27 [b]	10	No spread of infection took place from this fair to any district.
Ram Naumi fair, Ayodhya, March 1950	200 000	Compulsory	98 773	40.39 [c]	—	—	No spread of infection took place from this fair to any district.
Debi Patan fair, March-April 1950	100 000	Compulsory	64 959	65.00 [c]	—	—	No spread of infection took place from this fair to any district.
Kumbh fair Hardwar, March April 1950	1 200 000 (on the main day)	Compulsory	1 025 000	85.42 [d]	11 [b]	4	No spread of infection took place from this fair to any district.

Based on Table II of Gopal, 1951
[a] This figure, given by Gopal, seems clearly wrong
[b] All imported
[c] These figures refer only to pilgrims vaccinated at the approaches to the fairs, and do not include those who, having been vaccinated elsewhere, presented vaccination certificates.
[d] Exclusive of pilgrims vaccinated outside Uttar Pradesh

observers Thus Siler (1944) enumerated among other measures useful for cholera control

"limitation of the movements of individuals (potential or actual carriers) within epidemic areas and to and from such areas, so far as may be practical in the face of religious and social customs"

Similarly Napier (1951) advocated that when cholera appeared in villages in a non-endemic area cordons should be placed around them to prevent movements of the people In agreement with the impressions which Napier had gained in India Kamal (1951) declared that

"One of the biggest achievements exercised during the 1947 Egyptian epidemic, was the limitation of movements of the inhabitants. Railways, taxis, lorries, even private cars and cabs, individuals themselves, were all subjected to all forms of limitation of movement, sometimes complete, sometimes subject to written permits issued by health offices. No doubt this measure hit the economical and trade aspects of the country but it had its effect on the epidemic."

Most important was that, according to Kamal

"Upper Egypt escaped the heavy and severe spread of the disease due to that measure. It began by separating Upper from Lower Egypt completely for six days extended to ten, passenger trains were stopped and roads were guarded. Were it not for smugglers who travelled in a crooked way via untrodden paths, in the eastern hilly part of the Nile Valley Upper Egypt south of Giza province would have been free And notwithstanding this, the incidence of the disease in the southern provinces was very remarkably low if compared with Lower Egypt"

Since it is by no means invariably possible to take advantage of cordons or even lesser traffic restrictions and since moreover even if such measures can be used, they hardly ever function perfectly it is essential at times of cholera outbreaks to keep a close watch over the traffic routes leading out of the foci of the infection, particularly over railways and inland water routes so as to detect and isolate cholera patients or suspects It is most desirable that travellers who have not been vaccinated before obtaining permission to leave the foci (as ought to be the rule) be immunized against cholera at the inspection stations established at suitable points of the traffic routes

However indispensable though it is to take measures of this kind and to utilize cordons and other traffic restrictions as much as possible one should never lose sight of the fact that by far the most important means of checking the spread of cholera is drastic anti-epidemic action within the foci of infection

International Quarantine Measures

Introductory remarks

As alluded to in the preceding chapter the question whether or to what extent to impose quarantine measures against the spread of cholera

Local Quarantine Measures

Before proceeding to a discussion of the methods adopted in international quarantine work against cholera, some attention has to be paid to what may be called local quarantine measures, the aim of which is to prevent the spread of the infection from an affected community to those adjacent and along inland traffic routes

Dealing with the measures falling into the latter category Liebermeister (1896) made the following well reasoned statement

"The recognition that the existence of cholera depends upon the presence of specific bacilli, and that the latter cannot be transported over long distances through the air immediately leads to the theoretical conclusion that the propagation of the disease can be prevented through interruption of all communications [*Absperrung*]. [Trans.]

However Liebermeister continued,

"Opinions regarding the practical efficacy of measures of segregation and quarantine are divided. Not only those medical men who ascribe to the cholera bacilli only a subordinate rôle in the genesis of the epidemics, considering the local disposition to be of paramount importance, declare all measures of traffic interruption to be inefficacious and inappropriate, but, strangely also some adherents of the bacteriological school In doing so they can count upon the agreement of the merchant class, whose interests are severely damaged through any traffic interruption, as well of the public in general, as far as it is not overwhelmingly influenced by fear of the disease." [Trans.]

Liebermeister himself while fully realizing the difficulties confronting a complete system of segregation, pointed out that it had been successful whenever it could be strictly implemented For instance in 1831 cholera had remained absent from the Russian court (about 10 000 persons) owing to its complete isolation in Peterhof and Tsarskoye Selo Likewise St. Petersburg was free from cholera while it was surrounded by a military cordon in 1830 and early in 1831 and became invaded by the disease only in June 1831 when the troops were removed to combat the revolution in Poland On the other hand Liebermeister stressed, often simple and but little irksome measures of segregation and quarantine would have sufficed to prevent the spread of the infection Hence, he maintained,

"it would be most appropriate in this as in many other respects to make a compromise between theory and practice. Though it is true that it is only exceptionally possible to adopt a system of complete interruption of the communications, still this has to be considered as the ideal, to be approached as far as the circumstances permit. It is important at the time of cholera outbreaks to restrict traffic as much as can be done without detriment to important interests, when particularly assemblies of the population, like annual markets, festivals, pilgrimages and concentrations of troops are prevented." [Trans.]

It is curious to note that, while many subsequent writers did not share the opinion held by Liebermeister regarding the value of traffic restrictions and cordons, their use has been recommended once more by some modern

international defence against cholera had received some earlier attention in various ways It seems permissible in this connexion first to make some reference to the attempts, started as soon as the disease began to make inroads into Europe, to stop its progress with the aid of cordons As mentioned in Chapter I, some use of such cordons had been made already in 1823 by the Astrakhan authorities but in this case the cordons were probably placed inside Russian territories and not on the border However as described by Sticker (1912) when a new wave of the infection approached Russia in 1829,

"the medical council of the Russian Ministry of Interior had fortified the threatened frontiers with the means which it considered adequate and indispensable These comprised no less than a double line of troops along the frontier quarantine stations at all major roads, surveillance, washing, airing and fumigation of all that arrived from the infected countries, quarantine of all ships for up to 40 days obligatory reporting of each suspicious case, etc." [Trans.]

As further described by Sticker a similar programme was adopted by the countries neighbouring on Russia to the west. They used for this purpose the old Austrian and Prussian plague-cordon line

"to show to cholera *its official frontier This line contained at intervals of about 3000* paces highly situated guard-houses the intervals between these were filled by patrols. The guards had loaded rifles the officers were mounted. The guards had to watch day and night that neither human beings and cattle nor goods broke through the cordons. Everything that wanted to pass the frontier had to do so at certain stations, where quarantine periods and purification were imposed." [Trans.]

After the failure of the Russian cordons had become known even more Draconic measures were enforced, the cordons being doubled and drastic penalties, including even capital punishment, being envisaged for transgressions However as Sticker an ardent anti-contagionist, noted with obvious glee in a later section of his book, the infection paid no heed whatsoever to the cordons or to quarantines extended for twenty forty or even a hundred days and to disinfection and destruction by fire, but spread incessantly

It is interesting to note that this progress of cholera in Europe called not only for the above quarantine measures but, according to Howard Jones (1950) also for some efforts at international exchange of experience consisting first (early in 1831) of the dispatch of two medical men from Great Britain to St. Petersburg to study the manifestations of the disease Later in that year the great French physiologist Magendie went for the same purpose to England, and afterwards a committee of Italian physicians arrived from Rome in Paris But, as Howard Jones drily remarked "all these visits of investigation were fruitless"

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from an affected country to other countries formed one of the most important problems debated by the two opposed schools of the "contagionists" who urged the adoption of the most stringent regulations, and the "localists", who denied the need for any quarantine work However vociferous as the adherents of the latter school were in peremptorily propounding their extreme views for a long time the contagionists were able to overrule their adversaries and as a result a most rigid system of quarantine measures against cholera was enforced which it took more than a century to reduce to the reasonable programme recently adopted. Trying to offer an explanation of this slow progress, Gotschlich (1913) aptly stated

"When these exotic diseases [i.e., plague and cholera] appeared in Europe—especially in former times, when a rational system of prophylaxis was totally lacking—they usually raged in a terrifying manner causing tremendous losses in human lives as well as immeasurable economic damage thus, the less it was possible effectively to control an epidemic once it appeared within a country the more imperative it became to apply rigorous measures to ensure that the epidemics were kept away from the countries concerned." [Trans.]

To a philosophical mind it is curious to compare this statement with the text of an editorial appearing in *1947* in the *Lancet* wherein attention was drawn to unnecessarily drastic quarantine regulations imposed by some little endangered countries on the occasion of the 1947 cholera outbreak in Egypt. Pointing out that the flimsy foundations on which the prolonged efforts to adopt international quarantine regulations against cholera were based had thus been revealed, the editorial felicitously concluded by saying that

"Cholera has been called, in quite a different sense the disease of fear and certainly fear of it has caused incalculable trade and traffic losses which were largely needless. The lesson will have to be studied carefully when the time comes for the World Health Organization to revise the conventions. It seems clear however that it was not the conventions that were at fault, and that what we have witnessed was a partial return to the quarantine of the jungle."

In view of the almost universal adoption of up-to-date quarantine regulations promulgated by the World Health Organization in 1951 there is ground for hope that, should cholera ever again become more than the problem of local importance for the Far East which it now largely is, it will be reason and not panic which will govern the steps taken to avert a further spread of the infection.

History of cholera quarantine practices [1]

Though, as will be discussed soon it was only in 1851 that the representatives of several States met to hold a sanitary conference, the problems of

[1] This disquisition is mainly based upon the interesting article on the Origins of international health work by Howard-Jones (1950) and the more recent and exhaustive study on *Disease control and international travel* by Goar & Deutschman (1956). Attention is also drawn to the older and well-documented contributions of Gotschlich (1913, 1930) to the German *Handbook of pathogenic micro-organisms.*

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had been made as early as 1834 and again in 1843 it was not until 1851 that the first international sanitary conference was actually held at Paris. Thus as Goodman (1952) whom the two authors quote aptly stated

"For the first time, doctors and diplomatists officially representing twelve countries had met to discuss health problems. For the first time, an International Sanitary Convention had been drawn up embodying international rules to promote uniformity in quarantine procedure. For the first time, a number of important principles had been agreed, which were later embodied in international quarantine practice for example, the principle that quarantine was not applicable to ships with a clean bill of health and otherwise free from infection that maximum and minimum periods of quarantine were to apply differing for each of the three diseases dealt with that measures to ensure a healthy voyage should be taken at the port of departure that quarantine stations (lazarettos) should be provided for the performance of quarantine and that they should be hospitals rather than prisons and that epidemic intelligence reports from independent medical officers stationed in areas from which infection came were of great value and that this system should be extended "

Indeed, Goodman concluded, "fifty years of subsequent international discussion failed to alter the main principles of this first International Sanitary Convention" which thus marked the beginning of a new epoch in the field of health collaboration between governments.

Among the further early sanitary conferences enumerated by Gear & Deutschman, the following were of special importance as far as the control of cholera was concerned.

(1) The third conference of 1866 at Constantinople which, prompted by the importation of cholera by pilgrims into Egypt and its subsequent spread into Europe, devoted special attention to this disease, discussing its origin and propagation and deliberating upon control measures, including a special programme for the Mecca pilgrimage. As Gear & Deutschman added the conclusions reached were very accurate considering the fact that the conference took place seventeen years before the cholera vibrio was discovered.

(2) The 1874 conference convened, according to Gear & Deutschman,

"in Vienna, at the suggestion of Russia, which was concerned at the persistence of cholera in her territory and at the quarantine to which her maritime trade was subject because of the disease. Another reason for international examination of the problem of cholera at this time was the more frequent and faster traffic in the Red Sea which resulted from the opening of the Suez Canal in 1869"

Confirming on the whole the conclusions of the Constantinople meeting, the Vienna conference condemned land and river quarantine as useless. As far as maritime quarantine was concerned, a compromise solution was found, which provided for the choice of medical inspection or detention as measures to be applied to ships.

(3) The conference meeting in 1892 at Venice which sanctioned a system of limited protection in regard to the Mecca pilgrimages discussed earlier

at the Rome conference of 1885 The latter according to Gear & Deutschman

"In spite of British opposition supported to some extent by Denmark and the USA which had given up quarantine in favour of medical inspection recommended surveillance of 24 hours for healthy ships and a quarantine of three to six days for healthy persons in infected ships."

(4) The problems of cholera prevention again received much attention at the conferences held in 1893 and 1894 respectively in Dresden and Paris but, as noted by Sticker (1912) the usefulness of the recommendations made there was debated Thus when Kerschensteiner & Gaffky (1895) speaking at the 1894 meeting of the German Society of Public Health maintained that

"One must gratefully recognize the international endeavours to restrict cholera to its home and to prevent its propagation, and must do everything possible to promote the effective execution of the resolutions of Dresden and Paris [1893 and 1894]" [Trans.],

they were opposed by Koch who as quoted by Sticker stated that

"In regard to prophylaxis the convention of Dresden and the conference at Paris are of no use whatsoever The Dresden convention fulfilled its task by removing unnecessary traffic restrictions and was thus beneficial. The Paris conference tried to close certain portals of entry of cholera, especially the Red Sea and the Persian Gulf through quarantine and other supervisory measures. However with the exception of a single instance thus far cholera has reached us over the land route through Central Asia and not through the Persian Gulf or the Red Sea."[1] [Trans.]

(5) Noteworthy further progress in international cholera prevention was made at the 1903 conference in Paris As summarized by Masters (1947) the salient points of the convention then signed were as follows

"This convention substituted for the anarchy which had heretofore existed a co-operative scheme for combating epidemics based on the assumption by each signatory state of two important obligations (1) to notify all other signatories of the appearance in its territory of certain communicable diseases specified in the convention and (2) when so notified, to refrain from imposing against a stricken country defense measures in excess of the maximum permitted by the convention

"In addition to the above-mentioned principles which apply to all signatories, the 1903 convention contained special provisions for the Suez Canal and neighboring countries which were designed to establish under international administration a barrier against disease at strategic points on the Suez Canal, the Red Sea and the Persian Gulf it also provided detailed regulations for the Moslem pilgrimages "

The modern phase of the history of quarantine practices may be said to have begun in 1907 when, following an agreement signed in Rome, the Office International d Hygiène Publique according to Gear & Deutschman "the first truly world wide organization to deal with international health matters, especially with quarantine" was set up in Paris Through

[1] It would seem, however that, when making this statement, Koch failed to take due account of the important role played in the prevention of cholera inroads by quarantine stations like that of El Tor, the usefulness of which he had formerly acknowledged (see Gaffky & Koch, 1887 quoted by Wiener, 1913).

its information service and more still through the deliberations of its assemblies and committees the results of which were published together with many other important reports and reviews in a monthly bulletin, the Paris office played an invaluable role in taking the lead in international quarantine work until, after the Second World War its excellently fulfilled duties were taken over by the World Health Organization.

Reference has to be made also to an exhaustive study entitled *The prevalence of epidemic disease and port health organisation and procedure in the Far East*, made under the auspices of the League of Nations Health Committee by White (1923) on whose recommendations an epidemic information bureau was established by the Health Section of the League at Singapore This also functions now under the direction of the World Health Organization.

Further milestones on the road to the now adopted international quarantine practices were the signing of a new sanitary convention at a conference held in Paris in 1926 and of special sanitary regulations for aerial navigation at The Hague in 1933 As summarized by Gear & Deutschman,

"The 1926 convention, partly modified in 1938 represented the ultimate achievement of the movement begun in the nineteenth century for a compromise between contending medical theories and between these and the practical needs of international movement of people and goods."

The international sanitary convention for aerial navigation was based on the same principles as the general agreement made at Paris in 1926.

Before continuing with a discussion of the present status of international cholera quarantine, it seems indicated to pay separate attention to the evolution of some of the principles underlying this work.

Regulations of goods traffic

The labile status of the ideas held in regard to the measures considered necessary in order to prevent the spread of cholera through international traffic is well illustrated by the regulations successively adopted for the importation of goods from infected countries It was only after the discovery of the cholera vibrio that some authors dared to take a determined stand against the formerly adopted practices of either prohibiting all imports from affected localities or at least disinfecting or fumigating all articles brought in from such places Thus Koch declared at the 1884 cholera conference in Berlin that

" thus far cholera has never been brought to us through goods from India never were letters or other articles sent by mail responsible for the importation of cholera, even if they were not perforated [*durchstochen*] and fumigated, as is now frequently done." [1] [Trans.]

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[1] Well documented and excellently illustrated historical notes on the disinfection of mail have been published recently by Meyer (1952).

Flügge (1893) stated more explicitly that

"There is not the slightest reason to restrict the traffic in goods of all kinds and of mail if cholera approaches. According to the experimental investigations moist used linen and moistly kept foodstuffs are the only objects in which the comma bacilli can survive transport. All other goods, wool, cotton, artificial wool, hairs, tobacco paper even rags (which in wholesale trade are invariably sent in a dry condition) are completely innocuous Thus the results of bacteriological experiments justify at most an embargo on the importation of moist linen and foodstuffs." [Trans.]

However though these principles were adopted at the subsequent sanitary conferences (see, for instance Gotschlich 1913) it is melancholy to note that in some countries the panic created by the 1947 Egyptian cholera epidemic brought about a return to senselessly severe earlier practices including stoppage or disinfection of mails As mentioned in the 1947 editorial of the *Lancet* quoted earlier, one country even went so far as to prohibit all import of foodstuffs not only from Egypt but from eight other countries, because these were supposed to be threatened by the infection As will be discussed soon the 1951 international sanitary regulations definitely prohibited subjecting mail newspapers and the like to any sanitary measure, but prescribed or permitted reasonable action in the case of potentially contaminated articles including *inter alia* the bilge-water and other possibly dangerous water supplies of infected ships as well as suspected foodstuffs which were to be consumed raw

Control of the Mecca pilgrimage

Though as noted in earlier parts of this book, Mecca had been the scene of sometimes disastrous cholera outbreaks since 1831 it was only after the 1865 epidemic there which lead to a catastrophic spread of the infection that international attention was focused on the difficult problem of taking preventive action in regard to the annual pilgrimages to that holy place As stated above a programme of measures was adopted in this respect at the 1866 international sanitary conference in Constantinople It appears, however that the steps then envisaged were concerned with the prevention of further spread of cholera from the Hejaz into Egypt and other countries to the west rather than with the far more logical procedures of preventing the departure of infected pilgrims from the east and of reducing the risk of propagation of the disease at Mecca. In fact, as can be gathered from a valuable account on the Mecca pilgrimage and the measures adopted in regard to it contained in the report which Gaffky wrote in 1887 in co-operation with Koch it was only in the Netherlands East Indies that the authorities were able to let only those pilgrims depart who had sufficient funds for their pilgrimage To impose this or any other restriction on the pilgrims wishing to depart from India was considered incompatible with the tenets of religious toleration, and as a result many insufficiently clad and

badly nourished people left there who had just been able to scrape together their passage money but had to depend upon charity as soon as they arrived in the Hejaz. The herd immunity of the pilgrims was further lowered by (*a*) the comparatively large number of aged persons, many of whom indeed wished to die and be buried in Mecca and (*b*) the participation of numerous women and children in the pilgrimages

As described in the 1887 Gaffky Koch report, great efforts had been recently made to improve the sanitary conditions of Mecca and to supervise the health of the pilgrims, but as proved by the continued occurrence of cholera manifestations, these measures, the commencement of which went back to the *sixties* of the nineteenth century were far from giving fully satisfactory results

Referring to the quarantine activities to protect Egypt, the report written by Gaffky in co-operation with Koch stated that

"The cholera epidemic of the year 1865 and the international sanitary conference held in the following year at Constantinople led to the creation of a sanitary and quarantine service in the Red Sea, the organization of which was commenced in 1866 by the "Intendance Sanitaire d'Egypte" [sanitary administration of Egypt] created in the meanwhile. In the following year the necessary arrangements were made for quarantine stations at El Wedj and El Tor as well as for a station at the Springs of Moses "[1] [Trans.]

Use of these quarantine stations seems to have been made first in 1871 when,

"As soon as the first rumours of an outbreak of cholera in the Hejaz became available, the *conseil*[2] in Alexandria, in order to prevent a repetition of the events of 1865 resolved to subject the returning pilgrims to the regulation observation in El Wedj. Later the quarantine time was prolonged to 20 days in El Wedj and 10 days at the Springs of Moses. Egypt remained free from cholera on this occasion." [Trans.]

To judge from Gaffky s report use of the El Tor station was first made in 1877 78 and was continued on an increasing scale during the following years

A further important event taking place during this period was the opening, in 1881 of a quarantine station for the pilgrim ships coming from the east on the island of Kamaran in the Red Sea near the Yemen coast, which, however at first did not function in a satisfactory manner

The problems of the Mecca pilgrimage which as noted above, were again dealt with at the 1885 and 1892 sanitary conferences, received further exhaustive attention at the 1894 Paris conference in order to cope with the unsatisfactory functioning of the quarantine stations at El Tor and Kamaran and to give consideration to the proposals of Proust (1892)

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"soon after the cholera epidemic of 1902 the Department of Public Health realised that an organization for the control and prevention of cholera should be performed. A medical mission was attached to the pilgrimage to look after the pilgrims during their stay in the holy land. All pilgrims were inoculated against cholera before leaving to Hegaz. On their return they were put under quarantine at El Tor for three days where their luggage was disinfected and their faeces examined bacteriologically for cholera."

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"Pilgrims from areas where cholera was declared in the last 6 months must not be allowed to proceed to Hejaz unless they are put under observation for 5 days in the port of departure and no symptoms of cholera appear. In the meantime their stools must be examined bacteriologically and proved to be negative for cholera vibrios."

At the same time it was recommended that

"Pilgrims from areas where cholera was not declared during the last 6 months but where cholera is known to be endemic are not allowed to proceed on the voyage unless their stools are found to be free from cholera vibrios."

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However, as further stated by Khalil, these proposals were considered impracticable by the Indian health authorities and also did not meet with the approval of an international committee assembling at Alexandria in 1947 which resolved that

"Thanks to obligatory inoculation [against cholera] and to the period of medical supervision both before departure and during the voyage [of pilgrims], there has been no evidence of cholera in the Hejaz for many years.

"It seems unnecessary therefore to add to these apparently sound measures, a measure of doubtful value, *i.e.* the bacteriological examination of the stools.

"Further the mass bacteriological examination of stools necessitates a highly complicated organisation, a number of highly trained personnel and even then the result is *not likely to be reliable.*"

In agreement with these views, the International Sanitary Regulations issued by the World Health Organization in 1951 did not embody any more stringent stipulations regarding the control of the Mecca pilgrimage than the earlier conventions. More important still, as stated in a series of additional regulations issued by the World Health Organization in May 1956,[1] the Ninth World Health Assembly considering that there was no further need for the hitherto practised sanitary control of the Mecca pilgrimage,)[2] abrogated the relevant provisions of the 1951 International Sanitary Regulations Pilgrims being considered "persons taking part in periodic mass congregations" reference to their movement was made in Article 103 of the International Sanitary Regulations which, as amended read as follows

"1 Migrants, seasonal workers or persons taking part in periodic mass congregations and any ship, aircraft, train or road vehicle carrying them, may be subjected to additional sanitary measures conforming with the laws and regulations of each State concerned, and with any agreement concluded between any such States.

"2. Each State shall notify the Organization of the provisions of any such laws and regulations or agreement."

Though in the course of the disquisition on the control of the Mecca pilgrimage some reference had to be made to the use of stool examinations and of vaccination for the purpose of international quarantine against cholera, it is necessary before turning to a general appreciation of the 1951 sanitary regulations to deal with these two special subjects to some further extent.

Stool examinations in international quarantine practice

Owing to their nearness to the often cholera infected ports of China, the Philippines and Japan have in the past laid great stress upon stool examinations of all passengers, or at least all third-class passengers arriving

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[1] Reprinted in *Wkly epidem. Rec.* 1956 31 247

[2] As can be gathered from the report on the Ninth World Health Assembly in the *Chronicle of the World Health Organization* (1956), this decision was arrived at in view of the fact that a new quarantine station capable of dealing effectively with the pilgrim traffic had been formally opened at Jidda on 1 April 1956.

at a time when the disease was present on the mainland As claimed by White (1923) such a search for cholera carriers was first started in the Philippines in 1909 and seems to have been continued, whenever necessary, for many years Smith in 1938 for instance referred to the use of the method, which according to him was made obligatory by the United States quarantine regulations He recorded that among the 10 407 passengers whose stools were bacteriologically examined during the period from August to December 1937 296 (2.84%) proved to be carriers of *V cholerae* They were retained and treated with salol and Urotropine until daily repeated stool examinations had proved negative three times in succession.

At what time stool examination of potentially cholera-suspect ships passengers arriving from China in Japan was first used could not be definitely established. The earliest figures quoted by Iimura (1924) refer to the year 1916 Statistics for the period 1919 22 quoted by this author were as follows

Period of examination	*Number of faeces examined*	*Number of cholera patients discovered*	*Number of carriers*
July December 1919	120 637	8	11
August-October 1920	59 687	4	5
August-October 1921	8 091	1	3
August-October 1922	11 578	1	1

It has to be added that the Japanese authorities continued to lay great stress upon the stool examination of passengers arriving in their ports at the time of cholera outbreaks in China. In fact, as stated by Ide, in 1939 it was considered necessary to issue stricter quarantine regulations according to which the travellers, instead of being permitted to land after their stools had been collected were kept on board until the results of the bacteriological examinations had become known.

To judge from an article by Wiener (1913) at the El Tor station examination of the stools of all Egyptian pilgrims regardless of whether they were clinically suspicious for cholera, was made obligatory in 1911, whereas pilgrims from foreign countries intending to pass the Suez Canal were subjected to such tests only if they showed suggestive signs (as had been the general practice heretofore) As Wiener added, during the period from 1 December 1912 to 18 January 1913 the stools of over 14 000 pilgrims had been tested, but vibrios agglutinating with cholera immune serum had been isolated on only 69 occasions.

According to Crendiropoulo (1912) bacteriological examination of the stools of ships passengers was also used in 1911 and early in 1912 in the Alexandria quarantine station, when 34 361 such tests were made Vibrios were isolated upon 63 occasions, 23 of the strains proving agglutinable with cholera-immune serum As Crendiropoulo noted, true cholera vibrios were most often found in the stools of pilgrims who had left their country at the acme of an epidemic *pari passu* with the decrease of the outbreaks the

However, as further stated by Khalil, these proposals were considered impracticable by the Indian health authorities and also did not meet with the approval of an international committee assembling at Alexandria in 1947 which resolved that

"Thanks to obligatory inoculation [against cholera] and to the period of medical supervision both before departure and during the voyage [of pilgrims], there has been no evidence of cholera in the Hejaz for many years.

"It seems unnecessary therefore to add to these apparently sound measures, a measure of doubtful value, *i.e* the bacteriological examination of the stools.

"Further the mass bacteriological examination of stools necessitates a highly complicated organization, a number of highly trained personnel and even then the result is not likely to be reliable."

In agreement with these views, the International Sanitary Regulations issued by the World Health Organization in 1951 did not embody any more stringent stipulations regarding the control of the Mecca pilgrimage than the earlier conventions More important still, as stated in a series of additional regulations issued by the World Health Organization in May 1956,[1] the Ninth World Health Assembly considering that there was no further need for the hitherto practised sanitary control of the Mecca pilgrimage,)[2] abrogated the relevant provisions of the 1951 International Sanitary Regulations. Pilgrims being considered "persons taking part in periodic mass congregations" reference to their movement was made in Article 103 of the International Sanitary Regulations which, as amended, read as follows

"1 Migrants, seasonal workers or persons taking part in periodic mass congregations, and any ship, aircraft, train or road vehicle carrying them, may be subjected to additional sanitary measures conforming with the laws and regulations of each State concerned, and with any agreement concluded between any such States.

"2. Each State shall notify the Organization of the provisions of any such laws and regulations or agreement."

Though in the course of the disquisition on the control of the Mecca pilgrimage some reference had to be made to the use of stool examinations and of vaccination for the purpose of international quarantine against cholera, it is necessary before turning to a general appreciation of the 1951 sanitary regulations, to deal with these two special subjects to some further extent.

Stool examinations in international quarantine practice

Owing to their nearness to the often cholera infected ports of China, the Philippines and Japan have in the past laid great stress upon stool examinations of all passengers, or at least all third-class passengers arriving

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recorded in this connexion that among 1500 travellers who had passed through the Damascus Thora lazaretto "192 had been suspect and among these 84 had been considered cholera carriers i e., a general percentage of 5 38" Calculating and plotting the carrier incidence for each successive week from the end of August to the middle of October 1931, they found that there was

"a parallelism, with a lag of 15 days, between the graph of the epidemic at Basra and the graph of the percentage of healthy carriers found in the Damascus lazaretto" [Trans.]

In his important study on cholera carriers entering Ceylon from South India to which reference has been made already in the preceding chapter Nicholls (1935) recorded that from 1 January 1931 to 31 July 1934 he had examined stool samples from 85 558 recruited estate labourers and 15 238 other passengers en route to the island Vibrios agglutinable with cholera immune serum were isolated from these specimens on 84 occasions Furnishing details of these results, Nicholls noted that

"*V cholerae* was isolated on 36 occasions from 6,008 mixed samples from 58,506 estate labourers, that is one person in 1 627 was found to harbour this vibrio but *V cholerae* was isolated on 45 occasions from 27 052 samples from estate labourers examined singly that is one person in 601 by this method was found to harbour *V cholerae* [1] It is concluded that the difference between the figures 1 627 and 601 indicates the degree of error introduced by examining a number of samples mixed together"

He also admitted that the methods adopted for the isolation of vibrios have considerable limitations

"because the vibrios may be present in insufficient numbers for successful isolation from culture media inoculated with only 0 1 gramme of faeces. It is well known that failure to isolate the vibrio from a person may be followed by successful isolation from the same person on subsequent days and this was experienced in Ceylon during the outbreaks of 1925 and 1926 Therefore, whilst it has been shown definitely that one estate labourer in 601 and one passenger in 3,541 was a carrier it is certain that the number of carriers was much greater than this."

That occasional use of stool examinations continued to be made in international quarantine practice is proved by a report of Kopanaris (1947) according to which such tests had been made in the case of 662 persons detained at the time of the 1947 cholera outbreak in Egypt on a quarantine island in the port of Piraeus, Greece No carrier was detected

As alluded to when dealing above with the control of the Mecca pilgrimage the problem whether stool examination is a necessary or even a useful method in international cholera quarantine practice has been the subject of constant debate Those who considered it indispensable to resort to such tests naturally asserted the great importance of this procedure Thus Creel (1911) maintained that such bacteriological examinations

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[1] The number of other third-class passengers from whom single samples had been examined was 10 623 and 3 were cholera carriers, i.e., one in 3541

number of cholera carriers diminished, whereas that of individuals harbouring cholera like vibrios increased

It is noteworthy that in 1911—when, as noted in Chapter 1 cholera had become manifest in all parts of Italy—the method of examining the stools of passengers arriving from potentially or actually infected localities was utilized in various other ports also Thus Saccone (1911) reported that he had made such tests in the case of 1400 emigrants intending to leave the port of Naples and had thus found 12 carriers of *V cholerae* Some use of the method was also made in the Austrian coastal provinces by Markl (1912) who found on one of these occasions two cholera carriers among about 400 passengers and crew of a steamer

Among further observations on this point recorded without adequate details by Wiener (1912) the following may be quoted (*a*) three cholera carriers were detected among 5200 individuals examined at quarantine stations on the Russian German border (*b*) from 7388 persons, arriving on 30 ships from Baltic ports, *V cholerae* was isolated on seven occasions (*c*) out of 641 persons arriving in Lisbon from various ports in Italy Syria, and other countries, none harboured cholera vibrios in their stools, while these proved positive in 12 out of 755 individuals examined after their arrival from Madeira.

That the serious cholera situation in Italy in 1911 also caused repercussions in the United States of America is proved by the following statement of Creel (1911)

"In July 1911 the cholera epidemic in Italy reached such proportions, and so many cases of cholera developed among Italian immigrants en route to the US and among those detained after arrival in quarantine at New York and other ports on the Atlantic seaboard, that the quarantine regulations of the Federal Government relating to cholera were amended so that all steerage passengers from cholera-infected ports to this country should be held for bacteriological examination before being discharged from quarantine."

According to Creel, from the middle of July to the middle of November 1911 26 930 stool examinations made at the New York quarantine station revealed the presence of 27 carriers [1] He added that in the early summer months of 1911 the Italian authorities started a search for cholera carriers among the prospective immigrants and were successful in 40 instances Later on they also detained the immigrants for five days at the port of departure

As described by Jude & Millischer (1933) in the course of their campaign to protect Syria and Lebanon (then under French mandate) against inroads of cholera from Iraq they eventually resorted not only to the usual methods of quarantine but also to that of double stool examination of travellers coming from the infected region and later even to stool examination of all travellers proceeding from Baghdad to Damascus The two authors

[1] Quoting slightly different figures, Craster (1913) reported that stool examinations of 26 678 passengers led to the detection of 31 cholera carriers.

of the regulations, the validity of these certificates, for which a standard form was recommended

"shall extend for a period of six months, beginning six days after the first injection of the vaccine or in the event of a revaccination within such period of six months, on the date of that revaccination."

Thus, it will be noted the 1951 regulations were in agreement with the view formerly accepted by the Office International d Hygiène Publique that a single dose of cholera vaccine could be considered sufficient for the purposes of international quarantine Morgan (1934) while admitting that this was the case as far as travellers coming from a cholera infected locality were concerned urged that those intending to proceed to such a locality from a cholera free region ought to be given the best possible protection through two vaccine injections

General regulations for international cholera quarantine

Commenting on the general provisions made in the 1951 International Sanitary Regulations in regard to cholera Gear & Deutschman gave the following summary

"A ship is to be considered infected if on arrival, it has a case of cholera on board or if a case of cholera has occurred on board during the period of five days before arrival. It is regarded as suspected if a case of cholera has occurred on board during the voyage but there has been no fresh case during the five days before arrival

"An aircraft is to be considered infected if, on arrival it has a case of cholera on board and as suspected if a case has occurred on board during the voyage but the person has previously been disembarked.

"A ship or an aircraft which has come from an infected local area or has on board a person coming from such an area is to be regarded as healthy if on medical examination, the health authority is satisfied that no case of cholera has occurred on board during the voyage.

"On arrival of an infected ship or aircraft

"1 Any passenger or crew member who produces a valid certificate of vaccination against cholera may be placed under surveillance for a period of not more than five days reckoned from the date of disembarkation, and all others who disembark may be isolated for the same period.

"2 Any baggage of an infected person or suspect, and any other article (e.g. used bedding or linen) and any part of the ship or aircraft which is considered to be contaminated may be disinfected and any water carried on board which is considered to be contaminated may be disinfected and removed and the containers disinfected.

"3 Human dejecta waste water and waste matter and any matter which is considered to be contaminated must not be discharged or unloaded without previous disinfection. The safe disposal of all such material is the responsibility of the health authority

"A suspected ship or aircraft may be similarly disinfected, and any passenger or crew member who disembarks may be placed under surveillance for a period of not more than five days reckoned from the date of arrival.

"A ship or aircraft ceases to be regarded as infected or suspected when the measures required by the health authority in accordance with the above have been effectively carried out and any infected person has been removed and isolated.

"not only confer the maximum of protection to a non-infected country but they also work the least hardship to commerce and the travelling public. In lieu of the expensive, irksome detention for an uncertain period at quarantine, bacteriological examinations allow the release of the ship and a large majority of the passengers after a delay of only 24 to 48 hours, according to the number of passengers and that, too with greater safety than was attained by the old clinical standard of quarantine."

However the overwhelming majority of the cholera experts, including those in India and China who after all, could claim to be most familiar with the problems of this disease were not in agreement with this and similar appreciations of the method under review It is therefore not surprising that, as quoted by Jude & Millischer (1933) the Cholera Subcommittee of the Office International d Hygiène Publique, in marked contrast to the optimistic appraisal of Creel, passed in May 1926 the following resolution

"In spite of the manifest role of cholera carriers, the Subcommittee has unanimously recognized that the general measures of an international order directed against these carriers would be difficult to apply and are, all in all, of a debatable practical utility The technical difficulties of [stool] examination as well as the intolerable shackles thus put on the circulation of travellers are, in fact, out of proportion to the usefulness one could expect from the viewpoint of prophylaxis" [Trans.]

As noted above, an even more decisive vote against the usefulness of stool examinations in cholera quarantine work has been cast by a special committee meeting in 1947 (see page 974) Though it was concerned only with the control of the pilgrim traffic, in view of observations like for instance those of Nicholls (1935) one cannot doubt that the declaration of this committee against the method of examining the stools of persons undergoing quarantine against cholera have to be considered generally valid. Acceptance of this view is indicated in the 1951 International Sanitary Regulations Article 69 of which stipulated that

"1 No person shall be required to submit to rectal swabbing.

"2. Only a person on an international voyage, who has come from an infected local area within the incubation period of cholera and who has symptoms indicative of cholera, may be required to submit to stool examination."

Use of cholera vaccination in international quarantine work

Though, owing to the diversity of views held in regard to the efficacy of cholera vaccination not all authorities are convinced of the value of this prophylactic method in international quarantine work its implementation has been recommended in the 1926 sanitary convention the 1933 and 1944 sanitary conventions for aerial navigation as well as in the 1951 sanitary regulations. As will be detailed below the latter in agreement with the earlier conventions, provided that special privileges should be granted to travellers coming from an infected locality if they were in possession of a valid certificate of vaccination against cholera. As defined in Appendix 2

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"Surveillance of any suspect for a period of not more than five days reckoned from the date of arrival and disinfection as described above may also be applied on arrival of a train or a road vehicle in which a case of cholera has been discovered.

"Certain precautions must be taken with regard to food aboard an infected or suspected ship or aircraft, a train or road vehicle on which a case of cholera has been discovered, or any of these which has come from an infected local area the health authority may remove, or prohibit the unloading of fish, shellfish, fruit or vegetables which are to be consumed uncooked or beverages, unless they are in sealed containers and the health authority has no reason to believe that they are contaminated. Any such food or beverage which is removed must be safely disposed of"

CONCLUDING REMARKS

When trying to utilize the knowledge gained in regard to the prevention and control of cholera for forecasting the further incidence of the disease one cannot fail to perceive that conditions favourable for its prevalence have become increasingly limited. Many countries in Europe and America, once open to the inroads of this dread infection, can no longer fall under its sway because progress in sanitation particularly safe water supplies, has rendered them proof against its penetration Though at a slower rate, dictated by a lack of adequate funds constant progress in these directions is also being made in the countries not yet enjoying the full blessings of public health work. Of at least equal importance is that within recent years, in addition to this system of what one might call indirect defence against cholera, admirable progress has been made in its active control through proper management of the major pilgrimages in India, which in the past have played a preponderantly ominous role in the epidemic spread of the disease.

However while it has thus become permissible to contemplate the present cholera situation without alarm, one should beware of undue optimism For it must be realized that the system of cholera control at present adopted, besides being unable to deal in a really effective manner with the situation in the endemic areas depends upon the continued absence of any disequilibrium apt to lead to a breakdown of the anti-epidemic measures and the sanitary defences. One has to fear therefore that, should such a disequilibrium ever come about—and this would be apt to promote a spread of the infection from the endemic areas to hitherto cholera free regions through the uncontrolled movement of large population groups—cholera would once more become a major menace Whether or how soon it will be possible permanently to thwart this potential danger through radical anti-cholera campaigns in the endemic foci combined with area wide sanitary improvements remains to be seen

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ANNEX

Annex

EXAMINATION OF CHOLERA-SUSPECT STOOL SPECIMENS

In place of the instructions for the laboratory diagnosis of cholera by Ahuja and colleagues (1950, 1951) which were appended to the text of Chapter 7 when that was first published in the form of a separate article in the *Bulletin of the World Health Organization*, it seems well to reproduce here part of a new set of such instructions compiled, in consultation with an international group of outstanding experts, by Burrows & Pollitzer (1958) and also published in the *Bulletin*. It would have been redundant to reprint here the first part of that compilation, which deals in summary fashion with the methods of collecting and preserving specimens and of preparing culture media and reagents for cholera laboratory work, as ample attention has been paid to these topics in the main text of this book Only the second part of the new instructions, therefore—summarizing the methods of examining cholera-suspect stool specimens—is given below in a somewhat abridged form.

Examination of Specimens

The cholera vibrio is excreted in large numbers, often in practically pure culture, in the rice-water stools characterizing the early untreated stage of the disease and its isolation is relatively simple The proportion of vibrios found in stool specimens taken on the second day of the disease, i e after the onset of general symptoms, may be greatly reduced and make up as few as one colony in 100 growing up on directly inoculated solid media. Thereafter the vibrios may continue to be difficult to find on direct culture or may apparently increase in abundance in general they are disappearing rapidly by the sixth to seventh day

The administration of antibacterial drugs such as sulfonamides tetracyclines and chloramphenicol appreciably reduces the period over which positive cultures may be obtained, though the disease process is apparently unaffected

Thus two factors affect the successful isolation of *V cholerae* for diag nostic purposes—namely the stage in the disease during which the specimen is taken, and whether or not antibacterial substances have been administered for therapeutic purposes before the specimen is taken. In general the cholera vibrio is isolated in about 50% of cases when the specimen is taken during the acute stage of the disease and inoculated directly on to solid media, and isolation is successful in 90% 95% of cases when more than one specimen is taken and/or more than one culture medium is inoculated

Microscopic examination

Earlier workers attached considerable significance to the demonstration of morphologically characteristic *V cholerae* in Gram-stained, heat fixed smears of rice water stool or a flake of mucus Such direct microscopic examination is now regarded as of little or no value in part because *V cholerae* cannot be differentiated with confidence from cholera like vibrios or from coliform and related enteric bacilli on morphological grounds alone, and in part because *V cholerae* in such preparations fre quently shows a large proportion of atypical cells

Enrichment culture

Preliminary culture in fluid media to give a relative increase in the proportion of *V cholerae* over that of extraneous micro-organisms present in the specimen is often desirable, especially when the specimen is taken later than 48 hours after the onset of the disease and is essential to successful isolation of the micro-organism from specimens taken after four to six days The fluid media described in Chapter 7—namely peptone water (pages 533-536) bismuth-sulfite fluid medium (pages 539-542) and potassium tellurite medium (page 542)—may be used for this purpose

The amount of inoculum is inversely related to the numbers of *V cholerae* suspected to be present. For example a flake of mucus from a rice water stool may suffice, or the medium may be prepared at double strength and inoculated with an equal volume of faecal suspension. The enrichment culture may be incubated for a few hours only (as little as two hours in the case of specimens taken during the acute stage of the disease) or for as long as 6 or even 12 hours (in the case of specimens from convalescents) and when the incubation time is extended it is desirable to subculture at more than one time-interval. The vibrios grow rapidly in the form of a thin film on the surface of the medium and a loopful of this material is used as inoculum for culture on solid media

Isolation in pure culture

Agar media should be inoculated directly with specimens taken during the acute stage of the disease as well as with an inoculum from an appro-

priately incubated enrichment culture if the latter has been made One or more of the solid media favouring the growth of *V cholerae* described in Chapter 7—namely bile salt agar (pages 564 565) Aronson s medium (page 558) and modified Wilson & Reilly bismuth-sulfite agar (pages 565 566)—are used for this purpose

After 18 hours incubation *V cholerae* appears on bile-salt agar as small colonies 1 mm or less in diameter that are raised smooth and completely translucent and literally dewdrop-like in appearance thus being readily distinguishable from coliform and similar bacilli Colonies of cholera like vibrios and *Alcaligenes faecalis* are closely similar but may show a very slight opalescence

On Aronson s medium minute colonies of *V cholerae* appear as early as after 10 hours incubation and after 15-20 hours not only increase in size but also take on a bright red colour This coloration is not specific for *V cholerae* but is only indicative of fermentation of the sugars contained in the medium

On bismuth-sulfite medium *V cholerae* appears after 12 to 18 hours as yellowish-brown colonies that in the case of some strains may acquire a dark metallic lustre on continued incubation This appearance while characteristic is not specific for *V cholerae* in that *Proteus* species give closely similar colonies

Heat fixed smears should be prepared from the characteristic growth on one or another of these media and stained by Gram s method The colonial growth appearance coupled with the demonstration of the Gram negative curved rods characteristic of *Vibrio* morphology provides evidence consistent with the assumption that the micro-organisms are *V cholerae* but identification of the latter can be regarded as no more than presumptive at this point

Identification

The identification of *V cholerae* is based upon (*a*) its biochemical characteristics i e the fermentation of sucrose and mannose but not arabinose the reduction of nitrate to nitrite and the formation of indole from tryptophane to give the cholera red reaction and a negative Voges Proskauer reaction (*b*) its failure to haemolyse goat or sheep erythrocytes under appropriate conditions and (*c*) its agglutination in O group I antiserum.

A subculture of the colonially and microscopically typical *V cholerae* is prepared from the isolation plate culture and used to inoculate media for biochemical tests These include one tube each of the sugar broths sucrose mannose and arabinose a tube of peptone water for the cholera red test and a tube of glucose phosphate/peptone water for the Voges Proskauer test In addition a tube of isotonic Douglas broth is inoculated for the haemolysis test (see below)

Biochemical reactions The fermentations should be read after 18 24 hours incubation to avoid the late fermentation of arabinose that occurs with some strains The nitroso-indole reaction, or cholera red test, *is carried out by adding concentrated sulfuric acid, about 1 drop per ml* of culture to the peptone water culture after 24 hours incubation a positive reaction is indicated by the development of a crimson to ruby colour appearing more or less rapidly on the surface and then spreading to the whole of the mixture two to three hours after the addition of the reagents

The glucose/phosphate/peptone water culture is incubated for two to four days, although according to Taylor Pandit & Read (1937) positive reactions may be obtained with cultures incubated for 24 hours To 1 ml of the culture are added 0 6 ml of a 5% solution of α naphthol in absolute ethanol and 0.2 ml of a 40% solution of potassium hydroxide The reagents are added in the order indicated, and it is important to shake for about five seconds after the addition of each reagent. A positive reaction is indicated by the development of a crimson to ruby colour in the mixture two to four hours after addition of the reagents Standard procedures commonly specify that the test should be read not later than four hours, but some workers with *V cholerae* read the test as late as 24 hours afterwards

Haemolytic activity In assaying the haemolytic activity of *V cholerae* and related vibrios it is of primary importance to distinguish between haemolysis as observed on blood agar culture and the lysis of suspended erythrocytes in admixture with a suspension or culture of the micro-organisms Thus many strains of true *V cholerae* show the zones of complete clearing of β haemolysis around colonies on a blood agar plate while others do not, though neither lyse red blood cells in suspension under the conditions indicated below The apparent contradiction was resolved by van Loghem (1913) who established that the haemodigestion observed on blood-agar cultures and the haemolysis of suspended erythrocytes were *basically different processes*

The test for haemolytic activity of vibrios, or Greig test, is carried out by adding 1 ml of a 3% suspension of erythrocytes to 1 ml of either a 24-hour culture of the micro-organisms in isotonic Douglas broth or a suspension of the micro-organisms harvested from an agar culture in isotonic saline and containing about 2000 million vibrios per ml Of the two the broth culture is regarded as preferable Goat erythrocytes were used in the test as originally devised, but sheep erythrocytes are at least equally satisfactory if not preferable human red blood cells are not suitable for the haemolysin test for differentiating *V cholerae* from the haemolytic (to goat and sheep cells) El Tor vibrios The mixture is incubated at 37°C for two hours read, stored overnight in the refrigerator and read again A positive reaction is indicated by clearing of the red-cell suspension

and liberation of free haemoglobin The haemoglobin is frequently reduced and the haemolysis is usually not a complete sparkling haemolysis but the test can be read without difficulty

Agglutination Since *V cholerae* is agglutinated by O group I antiserum it is said to be "agglutinable" while vibrios of other O-antigenic specificity are "inagglutinable" This terminology is not to be taken to imply that serologically unrelated vibrios are not agglutinated in their homologous antisera

The agglutination test may be carried out as a rapid slide agglutination either using morphologically typical colonies taken from the isolation plate or growth from an agar-slant subculture. Alternatively the agglutination may be the usual tube titration, using a suspension of the micro-organisms in isotonic saline and serial dilutions of 2^n of antiserum

In the rapid slide test a loopful of isotonic saline and a loopful of antiserum, appropriately diluted as determined by prior test against known strains of *V cholerae* are placed side by side on a clean glass slide Bacterial growth from an agar culture is then suspended in the saline to give a heavy milky suspension, and this drop of suspension is then stirred thoroughly into the drop of antiserum A positive reaction is indicated by the development of a curdled appearance, usually within one to two minutes, which is apparent to the naked eye and if desired, may be examined under a hand lens or dissecting microscope It is essential that the test be controlled with a suspension of the bacteria in saline without antiserum. By the third day of the disease, R forms of the vibrio may be encountered that are spontaneously agglutinable in salt solution obviously this control should be negative In the event of saline agglutinable forms being found, it is often possible to obtain a stable suspension by reducing the salt concentration to 0.5%.

Bacterial agglutination in serial dilutions of antiserum in the tube titration to titres within a dilution or two of that obtained with homologous antigen are more dependable evidence of the serological identity of the organisms tested. Normal rabbit serum frequently agglutinates *V cholerae* in dilution as high as 1/50 and it is preferable that the serum dilution series begin at a 1/100 dilution. High titred sera prepared by hyperimmunization are more satisfactory than sera with agglutinin titres of 1 2000 or less. It is essential here also that saline control tubes be included in the titration. A suspension containing 2000 million vibrios per ml (1 mg (dry weight) per ml) corresponding approximately to 5 units of the International Reference Preparation for Opacity (Maaloe, 1955) is a satisfactory agglutinating antigen.

Either of the above methods may be used to type *V cholerae* as the Inaba or Ogawa serotype Such typing is not essential to the laboratory diagnosis of the disease but if desired is readily carried out by substituting absorbed antisera for the bivalent diagnostic serum.

Summary and evaluation

The characterization of *V cholerae* in terms of the foregoing tests may be summarized as follows

sucrose	mannose	arabinose	cholera-red	Voges-Proskauer	haemolysis
+	+	—	+	—	—

Vibrios conforming to this pattern are usually found to be of serological O group I Such agglutinable vibrios conform to the definition of *V cholerae* and the micro-organism may be regarded as identified

Two deviations from this pattern are encountered with some frequency First, in an appreciable proportion of cases, perhaps as much as 1%, vibrios are isolated from patients with clinically typical cholera in practically pure culture which may or may not differ culturally from *V cholerae* but which do not agglutinate in O group I antiserum Whether such organisms are etiologically related to the acute diarrhoeal disease or represent contamination in the cholera stool specimen has not been satisfactorily determined.

Secondly the vibrios isolated may conform in all respects to the characterization of *V cholerae* with the exception that they are haemolytic These are the so-called El Tor vibrios of O group I and the test for haemolytic activity thus assumes primary significance in their differentiation from *V cholerae* Such haemolytic forms because they are frequently present in surface waters may be met with as contaminants in human stools, most often those of healthy individuals But they have been found to be the specific etiological agent in acute epidemic diarrhoeal disease clinically indistinguishable from cholera, in Celebes. Haemolytic vibrios have also been found in India in connexion with acute diarrhoeal disease (Mukherji, 1955) Whether these El Tor vibrios are distinct pathogens differentiable from *V cholerae* or are atypical variants of the cholera vibrio has not yet been determined.

However this question may eventually be resolved, only those vibrios conforming to the pattern described above are generally regarded as *V cholerae* and reported as such.

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